UNIVERSIDADE DE LISBOA
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Functional Genomics applied to the study of resistance against Powdery Mildew in grapevine

"Documento Definitivo"

Doutoramento em Biologia
Especialidade de Biologia de Sistemas

Diana Margarida Alpoim de Andrade Pimentel

Tese orientada por:
Prof. Doutora Ana Margarida Fortes (Orientadora)
Prof. Doutor Antonio Granell (Co-orientador)

Documento especialmente elaborado para a obtenção do grau de doutor

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Documento especialmente elaborado para a obtenção do grau de doutor

Bolsa PD/BD/114385/2016

2020

Diana Margarida Alpoim de Andrade Pimentel foi bolseira de doutoramento no âmbito do programa doutoral BioSys – Sistemas Biológicos, Genómica Funcional & Integrativa (FCT/PD/00065/2012) da Faculdade de Ciências da Universidade de Lisboa, financiada pela Fundação para a Ciência e Tecnologia do Ministério da Educação e Ciência, Bolsa de Doutoramento PD/BD/114385/2016. Este trabalho foi também financiado pela Fundação para a Ciência e Tecnologia do Ministério da Educação e Ciência através do projecto PTDC/ASP-HOR/28485/2017 - "GrapeInfectomics".

Agradecimentos

Durante este percurso tive o privilégio de contactar com diversas pessoas e instituições que me permitiram, não só desenvolver este projecto, mas também crescer, tanto a nível científico e profissional, como pessoal.

Primeiro de tudo, quero agradecer à minha orientadora, Ana Margarida Fortes, por me ter dado a oportunidade de integrar este projecto, por ver qualidades em mim, enquanto investigadora, que nem sempre reconheci e por confiar nelas desde início. Todo este caminho foi uma aprendizagem constante. Não posso também deixar de agradecer ter-me proporcionado a oportunidade de conhecer novas realidades em diferentes laboratórios e de me pôr em contacto com diversos investigadores, cujo trabalho já vinha acompanhando.

Nestes quatro anos tive o enorme prazer de visitar três laboratório diferentes. O primeiro foi o *Plant Genomics and Biotechnology Lab*, IBMCP, Valencia, Espanha.

Quero agradecer ao meu co-orientador, Antonio Granell, por me receber no seu grupo de investigação, pelo seu exemplo enquanto investigador e por todo o seu contributo no desenvolvimento deste projecto.

A todos os membros do laboratório 2.10, Camilla, Silvia G., Joan, Flor, Sara, Alfredo, Francesco, Asun, e Diego Orzáez, agradeço o acolhimento e a amizade. Fora do laboratório 2.10 não podia deixar de agradecer ao Marco, Noel, Davide, Anna, Cristina e Juan Carbonell. Quero deixar um agradecimento especial à Silvia Presa, pela paciência e ensinamentos no laboratório e à María Ángeles por toda a preocupação e carinho, foi quase como uma segunda mãe.

A segunda experiência no exterior foi no *Applied Metabolome Analysis Lab*, Max Planck Institute of Molecular Plant Physiology, Potsdam, Alemanha. Quero agradecer ao Joachim Kopka por me receber no seu laboratório, pelo interesse no projecto e por todas as discussões sobre ele. Quero agradecer também ao Alexander Erban por me guiar neste novo mundo que era para mim a metabolómica. Um obrigado à Ines, que me orientou no laboratório, e ainda aos restantes membros do grupo, à Olga, à Bo Ing e ao Ioannis Agradeço ainda, do fundo do meu coração, à Tamires, companheira desta aventura, sem ti esta experiência não tinha sido tão maravilhosa. Obrigada por me apoiares, tirares dúvidas e cozinhares para mim! Estou à espera da tua visita este ano.

A última paragem foi no *Grapevine Genetics and Genomics Lab*, Institute of Grapevine and Wine Sciences, Logroño, Espanha. Agradeço ao José Martinez-Zapater a oportunidade de integrar o seu grupo por dois meses, foi uma grande aprendizagem. Nuria, obrigada por me

acolheres no teu espaço de trabalho com toda a tua boa disposição, por me pores a trabalhar em Linux, por partilhares comigo todo o teu conhecimento em análise do RNAseq e por me responderes a todas as perguntas. Yolanda, só tenho que te agradecer por tudo o que fizeste pela minha integração em Logroño e por me teres ido buscar quando tive o furo no pneu! Carol, a tua alegria é contagiante, não tenho palavras para te agradecer, obrigada por me abrires a porta do teu espaço e me fazeres sentir em casa. Quero ainda agradecer à Mercedes, Javier Ibáñez, Joseane, Jeróme e Pablo, foi um gosto conhecer-vos.

Já em Lisboa, só tenho muito a agradecer a todos os que fazem e fizeram parte do gabinete de bolseiros 2.1.49., Dario, Carolina Sampaio, Carolina Ferro Rodrigues, Elsa, Helena, João, Rita, Rute, e Tamiris, e ainda aos que nos visitaram por curtos períodos.

Quero agradecer em especial às "meninas": Teresa, parece que nos conhecemos desde sempre; Sara, companheira do "Bora passear? O que vamos fazer no fim-de-semana?"; Cláudia, que parece que não parte um prato e parte a loiça toda, e Alexandra, desculpa todo o bullying com os teus almoços. A todas, muito obrigada pela vossa amizade, apoio e conselhos. Para quando a próxima viagem?

Não podia deixar de agradecer ao Flávio, companheiro de laboratório, por todas as discussões sobre ciência e temas aleatórios. És das pessoas mais esforçadas que conheço.

Agradeço ainda à Professora Helena, à Professora Cristina, ao Fernando e à Susana por toda a atenção, disponibilidade e simpatia.

Um obrigada gigante às minhas extraordinárias colegas de doutoramento e de casa. Márcia, obrigada por ouvires todas as minhas dúvidas existenciais, dilemas, tristezas, alegrias, e teorias da vida e por me retribuíres bons conselhos que já aprendi que são para seguir à risca! Margarida, foi maravilhoso ver o quanto evoluíste. Joana, obrigada por seres um exemplo de força de vontade.

A todos os restantes membros da melhor edição do BioSys, Daniel, João, Rafael, Madalena, Mariana, Marta, Marina e Ana Rita, um grande obrigado. Sem vocês isto não tinha tido tanta graça.

Por fim quero agradecer às pessoas mais importantes na minha vida, a minha família. Aos meus pais, por todo o amor e apoio incondicional, sem ele não teria chegado até aqui. E ainda ao meu avô Pimentel, com quem venho a aprender que não se desiste nas adversidades, que nunca é tarde para aprender e que só ganhamos em perdoar.

Abstract

Grapevine (*Vitis vinifera* L.) is one of the most valuable non-climacteric fruit crops worldwide and is susceptible to several pathogens. Powdery Mildew (PM) is one of the most widespread diseases and is caused by the biotrophic fungus *Erysiphe necator*. This pathogen relies on the host metabolism to complete its life cycle. PM can affect all green tissues, such as leaves and green berries, frequently resulting in a negative effect on grape production. Despite the several studies performed so far, the mechanisms behind grapevine defense are very complex, and responses against PM remain unclear in infected fruits. In order to study the mechanisms involved in grape berries response to PM infection in the early stages of ripening, naturally infected and control grapes from 'Carignan' variety were collected at green (EL33) and *véraison* (EL35) stages and metabolic, transcriptomic and hormonal changes upon PM infection were analyzed. Results demonstrated that PM-susceptible grape berries were able to induce defense mechanisms and accumulate defense-associated metabolites, such as resveratrol, catechins, gallic acid, and long-chain saturated fatty acids, which could be explored as markers of infection at earlier ripening stages on field conditions. Induction of defenses was also previously observed in leaves, but certain responses seem to be organ-specific, such as the reprogramming of fatty acid metabolism and isoprenoid biosynthesis. This study was the first to quantify jasmonates' levels in PM infected berries suggesting an involvement of specific jasmonates in response to PM. These growth regulators are classically associated with response to necrotrophic fungi. Some *LATERAL ORGAN BOUNDARIES (LOB) domain* (*LBD*) and *GRAS* genes were responsive to powdery mildew and/ or modulated at *véraison* stage. LBD constitute a family of plant-specific transcription factors with important roles in several plant processes. In this work, a genome-wide analysis was performed to identify and map the *LBD* genes in the grapevine genome. Fifty *LBD* genes were identified and grouped in two classes. Expression profiling suggests the involvement of LBD transcription factors in grapevine development, berry ripening and stress responses. GRAS transcription factor family has also been reported as involved in multiple processes; however, their role in fruit ripening is poorly studied. Tomato *SlGRAS10* and its grapevine ortholog *VviPAT6* were previously suggested as putative regulators of fruit ripening in both climacteric and non-climacteric plants. In this work, preliminary studies and targeted mutagenesis using CRISPR-Cas9 technology were developed in order to study the role of SlGRAS10 in fruit ripening.

Keywords: Biotic stress responses, grapevine, powdery mildew, LBD transcription factors, GRAS transcription factors.

Resumo

A videira (*Vitis vinífera* L.) é uma planta de fruto com elevada importância económica e susceptível a vários agentes patogénicos. O oídio é uma doença que afecta a videira e é causada pelo agente *Erysiphe necator*, um fungo biotrófico que depende do hospedeiro para completar o seu ciclo de vida. O oídio pode afectar todos os tecidos verdes da planta, como por exemplo as folhas e os bagos verdes, e tem frequentemente um impacto negativo na produção de uvas, levando a uma redução do rendimento e da qualidade da uva. Vários *loci* de resistência foram identificados em videiras nativas da América do Norte e da China e cruzados com a espécie europeia. No entanto, a maioria dos híbridos não desenvolve as propriedades organolépticas tão apreciadas na espécie *V. vinífera*. Embora tenham sido realizados vários estudos, os mecanismos envolvidos na resposta ao stress biótico são complexos e pouco se sabe acerca da resposta ao oídio, principalmente em uvas infectadas. De forma a compreender melhor como se desenvolve a resposta das uvas à infecção com oídio nas fases iniciais do amadurecimento, foram colhidas uvas naturalmente infectadas e sem infecção (controlo) da variedade 'Carignan', uma das variedades mais susceptíveis, em duas fases do amadurecimento: fase verde (EL33) e fase de pintor ou *véraison* (EL35). Foram analisadas as mudanças que ocorreram em caso de infecção ao nível do transcriptoma, perfil metabólico e hormonal. Os resultados demonstraram que as uvas infectadas induziram as defesas, através da activação de genes associados à resposta a stress biótico, como *EDS1, PR-10, STS, R,* e vários genes codificantes de receptores de membrana, e acumularam metabolitos comummente associados à defesa, como por exemplo resveratrol, catequinas, ácido gálico e ácidos gordos saturados de cadeia longa. Estes metabolitos podem ser explorados no futuro como marcadores de infecção para aplicação no campo. Foi possível observar que a reprogramação transcricional e metabólica foi mais intensa na fase verde, quando os níveis de infecção tendem a ser mais elevados. A activação das defesas já tinha sido reportada em folhas; no entanto, certas respostas parecem depender do tipo de órgão, nomeadamente, a degradação dos ácidos gordos e a biossíntese de isoprenóides parece estar activada nas folhas, o que não se observou nos bagos. Também se observou a sobre-expressão de genes associados à biossíntese de fenilpropanóides, incluído fenólicos e antocianinas. Na fase verde, houve uma acumulação de antocianinas totais em resposta ao oídio, adiantando a acumulação normalmente associada à fase *véraison*. No entanto o oídio não teve impacto ao nível dos restantes parâmetros associados ao amadurecimento, como peso dos bagos e conteúdo de glucose, frutose e principais ácidos orgânicos. Para além de genes de defesa, vários genes *MLO,* normalmente associados à susceptibilidade, foram sobre-expressos em

bagos infectados. A análise transcricional permitiu ainda detectar transcritos do fungo nas uvas infectadas, revelando a expressão de diversos efectores. Curiosamente, grande parte dos transcritos que codificam efectores EKA-like foram detectados apenas na fase verde. Perante o perfil hormonal, houve uma acumulação de ácido salicílico em bagos infectados, o que era esperado uma vez que esta hormona é essencial na resposta a fungos biotrófico. No entanto também se observou uma reprogramação ao nível dos jasmonatos, comummente relacionados com a resposta a fungos necrotróficos, tanto pela sobre-expressão de genes associados à síntese de ácido jasmónico como pela acumulação de jasmonoil-isoleucina e do seu derivado hidroxilado, indicando um envolvimento também na resposta ao oídio.

Alguns factores de transcrição da família dos *LATERAL ORGAN BOUNDARIES (LOB) domain* (*LBD*) e dos *GRAS* foram modelados em resposta ao oídio e/ou na fase *véraison*. A família *LBD*, também designada por *ASYMMETRIC LEAVES2*-like (*ASL*), constitui uma família de factores de transcrição específica do reino vegetal caracterizada pela existência de um domínio LOB na cauda N-terminal e com papel regulador em diversos processos, como por exemplo na regulação do desenvolvimento de órgãos e pólen, regeneração celular, resposta as agentes patogénicos e metabolismo de antocianinas e do azoto. No entanto, o papel dos genes *LBD* no amadurecimento da uva, no desenvolvimento da videira e na resposta ao stress foi pouco estudado. Com base em estudos genómicos e de transcriptómica disponíveis em bases de dados públicas e utilizando ferramentas bioinformáticas é possível prever a função biológica putativa de uma família génica. Neste trabalho foi realizada uma análise extensiva dos genes *LBDs* na mais recente versão do genoma de videira, incluindo análises filogenéticas e de expressão em diversos tecidos, fases de desenvolvimento do fruto, e condições de stress. Foram identificados cinquenta genes *LBD* em 16 dos 19 cromossomas da videira e a análise filogenética revelou a existência de duas classes e nove subclasses, incluindo novas subclasses. Os resultados de expressão sugerem um envolvimento de vários genes *LBDs* no amadurecimento e na resposta tanto a stress biótico como abiótico, suportados na maioria dos casos pela análise dos promotores e por dados de co-expressão. No geral, estes resultados permitiram a identificação de genes candidatos para uma futura caracterização funcional com uma função putativa na regulação do amadurecimento, como por exemplo os genes *VviLBDIa3* e *VviLBD1c3*, cuja expressão aumenta e diminui com o amadurecimento, respectivamente, e da defesa, como é o caso dos genes *VviLBDId6* e *VviLBDIIa3* cuja expressão é aumentada após infecção com *Botrytis cinerea* e oídio.

A família de factores de transcrição GRAS é caracterizada pela presença de um domínio GRAS na parte C-terminal. Os membros desta família já caracterizados estão envolvidos em

vários processos, como sinalização, desenvolvimento da planta, resposta a condições de stress e interacções simbióticas. O papel destes factores de transcrição no amadurecimento dos frutos foi pouco estudado. No entanto, estudos de expressão desta família identificaram vários genes regulados diferencialmente durante o amadurecimento. Dentro destes, o gene de tomate (*Solanum lycopersicum*) *SlGRAS10* e o seu ortólogo em videira *VviPAT6* foram identificados como reguladores putativos do amadurecimento tanto em frutos climatéricos como em não-climatéricos. Neste trabalho foram realizados estudos bioinformáticos preliminares e desenvolvidas plantas mutantes *knock-out slgras10* através da tecnologia CRISPR-Cas9 para a caracterização funcional do gene *SlGRAS10.* A tecnologia CRISPR-Cas9 permite a edição localizada do genoma e foi aplicada ao tomateiro com uma elevada taxa de sucesso. O gene *VviPAT6* será caracterizado no futuro, no entanto iniciámos a caracterização do *SlGRAS10* devido à facilidade de transformação do tomate em relação à videira. A análise de sequências permitiu comprovar a existência do domínio GRAS na proteína SlGRAS10. A análise de expressão a partir de dados disponibilizados publicamente para as variedades MicroTom e Heinz 1706 permitiu reforçar a hipótese de envolvimento no amadurecimento. Para a criação dos mutantes *slgras10*, foram desenhados dois guideRNA que foram, respectivamente, agregados em dois constructos com a endonuclease Cas9 e o marcador de selecção de resistência à canamicina, NptII. Cotilédones de tomateiro foram transformados com estes constructos através de transformação estável por *Agrobaterium tumefaciens*, de onde foram regeneradas novas plantas. Adicionalmente foram também construídos dois constructos para sobre-expressão do gene sob o controlo de dois promotores, respectivamente: promotor CaMV 35S, que é um promotor constitutivo, e o promotor E8, cuja actividade está restringida ao fruto.

Os estudos aqui apresentados revelam dados importantes sobre a resposta da uva ao oídio nas fases iniciais do amadurecimento e também sobre o envolvimento de factores de transcrição, como *LBDs* e *GRAS*, na modelação do amadurecimento e da resposta ao stress. Estas descobertas poderão ser aplicadas no futuro não só em estudos funcionais como em melhoramento de plantas e de práticas agrícolas, e também no desenvolvimento de novas ferramentas de detecção precoce de doenças no campo.

Palavras-chave: Stress biótico, videira, oídio, factores de transcrição LBD, factores de transcrição GRAS.

De acordo com o disposto no artigo 24º do Regulamento de Estudos de Pós-Graduação da Universidade de Lisboa, Despacho nº 7024/2017, publicado no Diário da República – 2ª Série – nº 155 – 11 de Agosto de 2017, foram incluídos nesta dissertação os seguintes artigos:

1. **Pimentel, D.,** Fortes, A.M. (2020) Targeted genome editing using CRISPR-Cas9: applications in fruit quality and stress resilience. In: Tuteja, N., Tuteja, R., Passricha, N., Saifi, S. (Eds.), Advancement in Crop Improvement Techniques. Woodhead Publishing. (Published Date: 1st June 2020).

2. Grimplet, J.*, **Pimentel, D.***, Agudelo-Romero, P., Martinez-Zapater, J.M, and Fortes, A.M. (2017) The LATERAL ORGAN BOUNDARIES Domain gene family in grapevine: genome-wide characterization and expression analyses during developmental processes and stress responses. Scientific Reports 7: 15968. doi: 10.1038/s41598-017-16240-5.
*Ambos os autores contribuíram igualmente.

Para além dos artigos incluídos nesta dissertação, foram também desenvolvidos os seguintes artigos:

1. Breia, R., Conde, A., **Pimentel, D.**, Conde, C., Fortes, A.M., Granell, A., Gerós, H. (2020). VvSWEET7 Is a mono- and disaccharide transporter up-regulated in response to *Botrytis cinerea* infection in grape berries. Frontiers in Plant Science 10, 1753. doi: 10.3389/fpls.2019.01753

2. Fortes, A.M., Agudelo-Romero, P., **Pimentel, D**., Alkan, N. (2019). Transcriptional Modulation of Polyamine Metabolism in Fruit Species Under Abiotic and Biotic Stress. Frontiers in Plant Science 10, 816. doi: 10.3389/fpls.2019.00816

3. Coelho, J., Almeida-Trapp, M., **Pimentel, D**., Soares, F., Reis, P., Rego, C., Mithöfer, A., Fortes, A.M. (2019). The study of hormonal metabolism of Trincadeira and Syrah cultivars indicates new roles of salicylic acid, jasmonates, ABA and IAA during grape ripening and upon infection with *Botrytis cinerea*. Plant Science 283, 266–277. doi: 10.1016/j.plantsci.2019.01.024

Contents

Abbreviations

4CL	4-coumaroyl-CoA ligase
ABA	Abscisic acid
alc	*alcobaça*
ANR	Anthocyanidin reductase
ANS	Anthocyanidin synthase
AOS	Allene oxide synthase
ARK3	Arabidopsis Receptor Kinase 3
ASL	Asymmetric leaves2-like
BAK1	Brassinosteroid insensitive 1-associated receptor kinase 1
C3H	p-coumarate 3-hydroxylase
C4H	*trans*-cinnamate 4-monooxygenase
Cas9	CRISPR-associated protein 9 nuclease
CAZymes	Carbohydrate-Active enZymes
CCOMT	Caffeoyl-CoA O-methyltransferase
CHI	Chalcone isomerase;
CHS	Chalcone synthase
cis-OPDA	12-oxo-phytodienoic acid
CLV1	Clavata1 receptor kinase
CNGC	Cyclic nucleotide-gated ion channel
COMT	Caffeic acid O-methyltransferase
COOH-JA-Ile	Dicarboxyjasmonoyl-isoleucine
CPK13	Calcium-dependent protein kinase 13
CREs	*cis*-regulatory elements
CRISPR	Clustered Regulatory Interspaced Short Palindromic Repeats
CRK10	Cysteine-rich RLK10
crRNAs	Small interfering CRISPR RNAs
CSEPs	Candidates for Secreted Effector Proteins
DAMPs	Damage-associated molecular patterns
dCas9	Catalytically inactive Cas9
DFR	Dihydroflavanol 4-reductase
DSBs	Double-strand breaks
EDS1	Enhanced Disease Susceptibility1
EKAs	Effectors homologous to Avrk1 and Avra10
EL33	Green stage
EL35	*Véraison* stage
ERF	Ethylene-responsive factors
ET	Ethylene
ETI	Effector-triggered immunity
F3’H	Flavonoid 3-monooxygenase
F3’5’H	Flavonoid 3',5'-hydroxylase
F3H	Flavonone 3-hydroxylase
F3'H	Flavonoid 3'-monooxygenase

F5H	Ferulate 5-hydroxylase
FLS	Flavonol synthase
FLS2	FLAGELLIN-SENSITIVE 2
FRK1	FLG22-induced receptor-like kinase 1
GA	Gibberellin
GABA	γ-aminobutyric acid
GC-EI/TOF-MS	Gas chromatography coupled to electron impact ionization/time-of-flight mass spectrometry
GMOs	Genetically modified organisms
GSTs	Glutathione S-transferases
HCBT	Anthranilate N-benzoyltransferase
HDR	Homology direct repair
HR	Hypersensitive response
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
indels	Insertions or deletion
JA	Jasmonic acid
JA-Glucoside	12-O-glucoside-jasmonic acid
JA-Ile	Conjugate jasmonoyl isoleucine
LAR	leucoanthocyanidin reductase
LBD	LATERAL ORGAN BOUNDARIES Domain
LOB	LATERAL ORGAN BOUNDARIES
LOX	Lipoxygenases
LRR-RKs	Leucine-rich repeat receptor kinases
LysM	Lysine motif
MAMPs	Microbe-associated molecular patterns
MAPK	Mitogen-activated protein kinase
MeJA	Methyl jasmonate
MeSA	Methyl salicylate
MLO	MILDEW RESISTANCE LOCUS O
MS medium	Murashige and Skoog medium
NBS	Nuclear binding site
NCED	9-*cis*-epoxycarotenoid dioxygenase
NHEJ	Non-homologous end joining
nor	*non-ripening*
NPBTs	New Plant Breeding Techniques
Ogs	Oligogalacturonic acids
OH-JA-Ile	Hydroxyjasmonoyl-isoleucine
OIV	International Organization of Vine and Wine
PAD4	Phytoalexin deficient4
PAL	Phenylalanine ammonia-lyase
PAM	Protospacer-adjacent motif
PAMPs	Pathogen-associated molecular patterns
PCD	Programmed cell death

PM	Powdery mildew
PR	Pathogenesis-related proteins
PRR	Pattern-recognition receptor
PTI	PAMP-triggered immunity
R genes	Resistance genes
RFNs	RNA-guided FokI nucleases
rin	*ripening inhibitor*
RING finger protein	Really Interesting New Gene finger protein
RKs	Receptor kinases
RLKs	Receptor-like kinases
RLPs	Receptor-like proteins
RNPs	sgRNA-Cas9 ribonucleoproteins
RTSK	Receptor serine/threonine kinase
S genes	Susceptibility genes
SA	Salicylic acid
SAG101	Senescence associated gene101
SA-Glucoside	Salicylic acid-β-D-glucoside
Sen1	Susceptibility to Erysiphe necator 1
sgRNA	Single guide RNA
sNCGGa	Super-Nomenclature Committee for Grape Gene Annotation
SPME-GC-EI-MS	Solid-phase microextraction and gas chromatography coupled to electron impact
STS	Stilbene synthase
TALENs	Transcription activator-like effector nucleases
TFs	Transcription factors
TMT	Tonoplast Monosaccharide Transporter
TPR1	Topless-related 1
tracrRNA	Transactivating CRISPR RNA
UF3GT	UDP-glucose:anthocyanidin 3-O-d-glucosyltransferase.
USDA	United States Department of Agriculture
WT	Wild type

Chapter 1

Introduction

The information reviewed in Section 1.4 will be published as a book chapter:

Pimentel, D., Fortes, A.M. (2020) Targeted genome editing using CRISPR-Cas9: applications in fruit quality and stress resilience. In: Tuteja, N., Tuteja, R., Passricha, N., Saifi, S. (Eds.), Advancement in Crop Improvement Techniques. Woodhead Publishing.

1.1. Grapevine, economic relevance, and grape berry ripening

Grapevine (*Vitis vinifera* L.) is one of the most valuable fruit crops worldwide, and wine production follows human history for about 8000 years ago (McGovern et al., 2017). This species belongs to the Vitaceae family, which includes around sixty inter-fertile *Vitis* species distributed mainly in the North Hemisphere under subtropical, Mediterranean, and continental-temperate climatic conditions (Terral et al., 2010). *V. vinifera* is the species with the most economical value due to its extensive use in the global wine industry (This et al., 2006). Other *Vitis* species, such as the North American *V. rupestris*, *V. berlandieri*, and *V. riparia*, are used in viticulture as breeding rootstocks, due to their resistance to several pathogens. Besides wine production, grapes are consumed fresh or dehydrated (raisins), used in the production of juice and distilled alcoholic beverages, and by-products are being used, especially the seed extract, in the nutraceutical and cosmetic industry due to its medicinal properties (Conde et al., 2007).

Cultivated *V. vinifera* ssp. *vinifera* (or *sativa*) derived from the wild populations of *V. vinifera* ssp. *sylvestris* and the two subspecies were distinguished based on morphological differences (This et al., 2006; Zohary, 1995). The domestication process involved the shift from dioecious to hermaphrodite genotypes, an increase in sugar content, berry enlargement, larger cluster size, and modification of seed morphology (This et al., 2006). Based on chlorotype variation and distribution of wild and cultivated grapes, at least two origins of Euroasian grape domestication are suggested for the cultivated germplasm, one in the Near East and another in the western Mediterranean region (Arroyo-García et al., 2006). European grapevines were introduced in America in the 16th century, and in South Africa, Australia, and New Zealand in the 19th century. Thousands of grapevine cultivars exist, but only a small group dominates the wine production. Grapevine is a diploid plant with an estimated genome size of approximately 500 Mb arranged in 19 chromosomes (Jaillon et al., 2007), and several genomic resources are available for *V. vinifera* species.

According to the OIV, 73.3 mt (millions of tonnes) of grapes were produced in 2017 in a total of 7.5 mha (millions of hectares) worldwide, being China the largest producer, followed by Italy, USA, France and Spain (OIV Report 2018). In the same year, 248 mhL (millions of hectoliters) of wine was produced globally, with Italy, France, and Spain heading the top producers. In Portugal, the wine industry has a significant impact on the national economy, accounting for around 700 million euros per year in exportation. In 2017, the vineyard area, and grape and wine productions were 193 672 ha, 896 089 t, and 6.7 mhL, respectively (OIV Report 2018).

Grape berry is a non-climacteric fleshy fruit formed by seeds surrounded by three tissue layers: endocarp, mesocarp (or pulp), and exocarp (or skin). Berry development follows a double sigmoidal growth pattern with three distinct stages, which includes two periods of growth separated by a lag phase (Conde et al., 2007). Coombe (1995) developed a new system for identification of grapevine growth stages, the Modified E-L system, which has been used as a reference. The first stage of the berry growth corresponds to the green stage (from EL27 to EL33), and it is characterized by rapid berry growth through cell division and expansion. Berries are firm and green due to the chlorophyll content. In this stage, organic acids are accumulated, and tannins, hydroxycinnamates and phenolic compounds precursors are synthesized. The first stage is followed by a lag phase when the growth slows down. At the end of this stage, a second growth stage initiates corresponding to the onset of ripening, also known as *véraison* (EL35). This second growth stage (from EL35 to EL38), where growth results exclusively from cell expansion (Coombe, 1976), is characterized by sugar accumulation, berry softening, a decrease of chlorophyll content, pigmentation of colored varieties through anthocyanin accumulation in the skin, and a decrease of the organic acid levels (Conde et al., 2007). Hormones play important roles in the regulation of berry ripening; abscisic acid (ABA), brassinosteroids, and ethylene have been suggested as ripening promoters, on the other hand, auxins, cytokinins, gibberellins, and jasmonates have been reported as inhibitors of ripening (Fortes et al., 2015).

1.2. Powdery Mildew

The Eurasian grapevine is susceptible to several pathogens, including fungi, oomycetes, bacteria, viruses, phytoplasma, insects, and arachnids. Pathogens can be classified as necrotrophic, biotrophic, and hemibiotrophic according to their life cycle and infection strategies (Glazebrook, 2005). Necrotrophs cause necrosis of the host tissues by secreting lytic enzymes and phytotoxins and/or hijacking the plant's enzymatic machinery (Glazebrook, 2005). Biotrophs depend on the host living tissue and develop strategies to invade the cell and obtain nutrients without inducing plant defenses (Glazebrook, 2005). Hemibiotrophs start with a biotrophic infection phase and then switch to a necrotrophic phase at different developmental phases (Glazebrook, 2005).

Powdery Mildew (PM) is one of the most widespread grapevine diseases and is caused by the biotrophic fungus *Erysiphe necator* Schw. [syn. *Uncinula necator* (Schw.) Burr.]. *E. necator* belongs to the Erysiphaceae family within the Ascomycota division and is an obligate pathogen of several genera within the Vitaceae family (Pearson and Gadoury, 1992). *E. necator*

genome is exceptionally large, accounting around 126 Mb, highly repetitive compared to other fungal plant pathogens, and was estimated to contain 6533 protein-coding genes (Jones et al., 2014). *V. vinifera* is highly susceptible to PM. As a biotrophic fungus, *E. necator* relies on host photosynthetic cells to complete its life cycle. This fungus was accidentally introduced into Europe around 1845 from North America, causing significant losses in vinicultural production. *E. necator* spread to other regions around the world, requiring the adaptation of vinicultural practices.

Powdery mildew is usually controlled with periodic foliar application of organic or inorganic fungicide during each growing season. Nevertheless, integrated disease management programs also include canopy management techniques. Sulfur is the most used fungicide due to its efficacy, reduced cost, and lack of pathogen resistance. A report on the use of fungicides in the European Union from 2001 to 2003 revealed that approximately 70% of the fungicides used were applied in vineyards; nevertheless, vineyards occupied only approximately 8% of the total crop production area (Phytowelt GmbH for the European Commission, 2003). This intensive use of fungicides could have a negative impact on the environment. Additionally, *E. necator* has been reported to evolve resistance to several fungicides (Dufour et al., 2011). Therefore, the development of grapevines with higher genetic resistance to pathogens would be crucial for sustainable grape production.

PM infection often has a negative effect on grape production, leading to decreased yields and affecting fruit quality, although the actual effect is controversial. Ough and Berg (1979) reported that wines from infected berries had slightly higher soluble solids. On the other side, Gadoury et al. (2001) observed that PM infection of *V. labruscana* 'Concord' vines had a negative effect on berry sugar levels, juice color and acidity. Calonnec et al. (2004) reported that PM infection of *V. vinifera* 'Cabernet Sauvignon' and 'Sauvignon blanc' led to a reduction in yield, berry size, and anthocyanin content, and an increase in sugar content and acidity Anthocyanin levels on *V. vinifera* 'Sangiovese' berries and wine were decreased upon infection (Amati et al., 1996; Piermattei et al., 1999). Wines produced with infected grapes were observed to contain more hydroxycinnamic tartaric esters (Amati et al., 1996). Total phenols and anthocyanins were also reduced in wines from infected berries; nevertheless, the content of *trans*-resveratrol, caftaric, and coutaric acids was significantly higher (Piermattei et al., 1999).

1.2.1. Symptoms of infection

Powdery mildew can affect all green tissues of the host, such as leaves, stems, inflorescences, and green berries. Macroscopically visible powdery mildew colonies create a white-grayish powder on the surface of infected tissues. On leaves, colonies are commonly developed on the lower surface of the young leaves, and at the upper surface chlorotic spots could be found. Colonies can also be found on the upper surface and appear whitish and with a metallic sheen. Heavily infected leaves may become senescent and falling prematurely (Gadoury et al., 2012). Leaves are more susceptible when half expanded, and older leaves often became more resistant to infection (ontogenic resistance); however, total immunity is not developed (Doster, 1985). Leaf ontogenic resistance correlates with the leaf transition state from source to sink (Calonnec et al., 2018).

Inflorescence and berries also present a whitish powder coating the surface. *E. necator* severe infection stops the growth of the berry epidermal tissue, leading to berry cracking during expansion (Gadoury et al., 2012). Ontogenic or age-related resistance is also observed in berries, and the period of susceptibility is relatively short, lasting up to 1-2 weeks after fruit set (Gadoury et al., 2003). Ontogenic resistance is observed in *V. vinifera* cultivars around 2-4 weeks after bloom (Ficke et al., 2004, 2003; Gadoury et al., 2003). Ficke and co-workers (2003) reported that during berry development, the rate of penetration, the formation of haustoria, and the development of secondary hyphae were reduced on older fruits; however, the conidial germination and appressorium formation were unaffected. During the transition between susceptible and resistant stages, berries develop diffuse mildew colonies, which are associated with infection with *Botrytis* bunch rot, increased population of epiphytic microorganisms and insects, and poor wine quality (Gadoury et al., 2007). Berries from some North American *Vitis* species exhibit constitutive resistance during all berry development (Gee et al., 2008).

1.2.2. Infection strategy

Erysiphe necator relies on the host metabolism to develop, growing epiphytically on the epidermis of the host green tissue and forming a dense white mycelium. During winters, mycelium with haustoria and conidia can stay dormant in infected lateral buds, giving rise to conidia-bearing flag shoots in the following spring (Gadoury et al., 2012). *E. necator* also overwinters as chasmothecia (formerly known as cleistothecia), which are sexual reproduction structures containing ascospores. During spring, ascospores are released from the chasmothecia and attached to the surface of the host plant and germinate like conidia. Free water is necessary to initiate discharge of the ascospores from chasmothecia, and ascospore infection is favored

by precipitation in excess of 2-3 mm together with temperatures above 10°C (Gadoury and Pearson, 1990a, 1990b).

During infection, both conidia and ascospores land on the epidermis of the host tissues and germinate, forming a primary germ tube that differentiates into a lobed appressorium, which is a specialized infectious structure. A penetration peg is developed from beneath the appressorium that creates mechanical pressure and enzymatic degradation of the cuticle and cell wall in order to penetrate the host cell wall (Rumbolz et al., 2000). In successful infection, a specialized feeding structure is formed, called haustorium, by which the fungus exchange molecules with the host cells; the fungus uptake hexoses, amino acids, vitamins, and other nutrients from the host cells, and secrete proteins to suppress host defenses (Qiu et al., 2015). To spread the infection, the fungus develops secondary hyphae along with the tissues, creating more appressoria and haustoria and developing asexual reproductive structures: conidiophores and conidia. Conidia are then released, and a new cycle of infection begins (Figure 1.1).

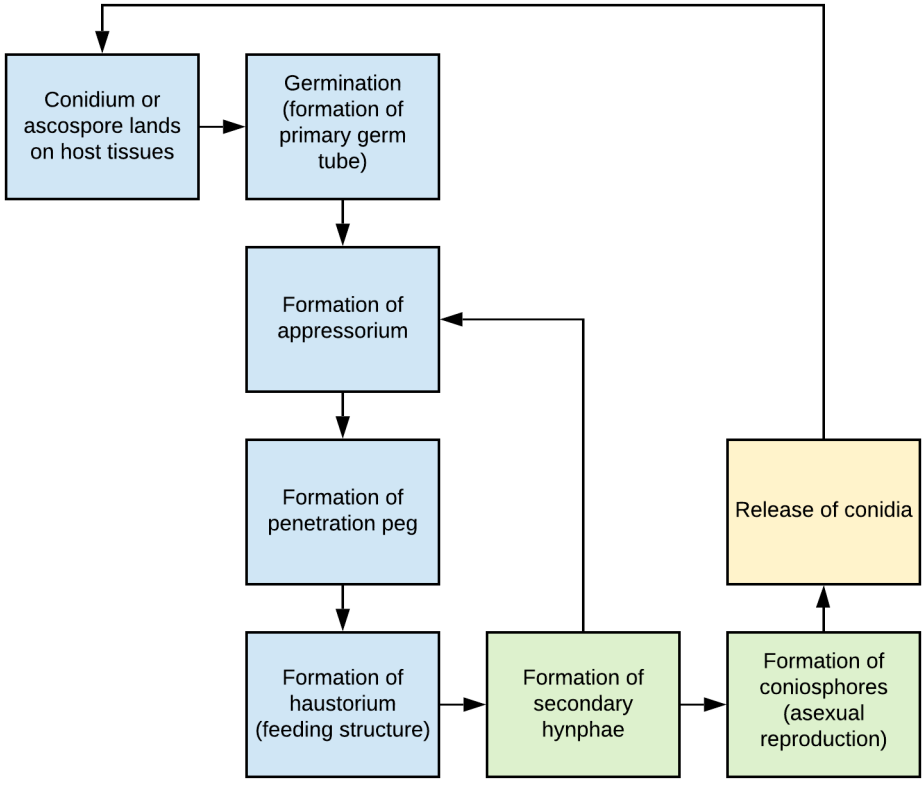

Figure 1.1 – *Erysiphe necator* infection strategy. First, conidia and/or ascospores land on host tissues and germinate, forming a primary germ tube. A lobed appressorium is formed. A penetration peg is developed from beneath the appressorium. In successful infection, an haustorium is formed. To spread the infection, the fungus develops secondary hyphae along the tissues, creating more appressoria and haustoria and developing conidiophores (asexual reproductive structure). Conidia are then released, and a new cycle of infection begins.

Environmental conditions regulate the growth and the spread of the pathogen. The principal factor is temperature. Powdery mildew colonies grow and sporulate faster, reducing the latent period, under temperatures from 23 to 30°C, with an optimum of 26°C (Delp, 1954). Conidia germination is inhibited at 35°C and killed after sufficient exposure to higher temperatures (Delp, 1954). Other relevant factors are atmospheric humidity and sunlight exposure. Powdery mildew develops better under a relative humidity of approximately 85%, and severity and incidence decrease as the air becomes drier (Carroll and Wilcox, 2003). Naturally and artificial shading from direct sunlight increase disease severity, partly as a result of the UV light filtration and the decreased leaf temperature (Austin and Wilcox, 2012). UV-B radiation reduces conidia germination and inhibits colony establishment (hyphal formation), and prolongs the latent period (Austin and Wilcox, 2012; Willocquet et al., 1996). Direct sunlight can increase the leaf surface temperature to an inhibitory or lethal level (Austin and Wilcox, 2012).

1.3. Biotic stress response and plant triggered immunity

As sessile organisms, plants developed several defense mechanisms to prevent pathogen penetration and colonization. Penetration resistance and programmed cell death (PCD)-mediated resistance are the main strategies to prevent fungal invasion. Penetration resistance blocks the entrance across the cell wall and plasma membrane preventing the formation of the haustorium, and PCD-mediated resistance occurs after penetration and induces the death of the invaded cells, disrupting the supply of nutrients to the fungus (Qiu et al., 2015). The two primary innate immune responses are the pathogen-associated molecular patterns (PAMP)-triggered immunity (PTI), mainly related to penetration resistance, and effector-triggered immunity (ETI), related to the restriction of pathogen growth and proliferation (Jones and Dangl, 2006). Both responses happen consecutively and are interconnected (Jones and Dangl, 2006). Figure 1.2 depict schematically the activation of the plant innate immunity system.

PTI, also called basal immunity, is the first line of defense and is activated by the recognition of pathogen-specific molecules, also known as PAMPs, by extracellular pattern-recognition receptors (PRRs) located at the plasma membrane of the host cells (Boller and Felix, 2009). PAMPs are also referred to as microbe-associated molecular patterns (MAMPs) since they are not limited to pathogenic organisms. In addition, affected cells can release endogenous damage-associated molecular patterns (DAMPs), which can act as endogenous elicitors (Boller and Felix, 2009). Activation of PRRs leads to activation of MAPK (mitogen-activated protein kinase) cascades, transcriptional induction of defense-related genes, such as

pathogenesis-related (PR) protein (e.g. β-1,3-glucanases and chitinases) and genes related to phytoalexin biosynthesis (e.g. resveratrol biosynthesis), production of reactive oxygen species, Ca^{2+} influx, activation of hormonal signaling, and cell wall reinforcement through deposition of callose (Meng and Zhang, 2013). Transcriptional induction of PTI confers resistance to a broad range of non-adapted pathogens (Boller and Felix, 2009; Jones and Dangl, 2006). PAMPs can be structural components of the pathogen, such as bacterial flagellin, lipopolysaccharides, peptidoglycans, fungal chitin, or oomycete β-glucans, or also secreted toxins or enzymes, such as fungal xylanase or endopolygalacturonase (Héloir et al., 2019). A large number of receptors have been identified for different PAMPs from bacteria and fungi. PPRs are typically receptor-like kinases (RLKs) or receptor-like proteins (RLPs) with an extracellular domain for PAMP recognition, including leucine-rich repeat receptor kinases (LRR-RKs) and lysine motif (LysM) kinases.

In Arabidopsis, the flagellin receptor FLS2 (FLAGELLIN-SENSITIVE 2), which is an LRR-RK, recognizes the highly conserved N-terminus of bacterial flagellin (flg22), inducing the expression of defense-related genes (Chinchilla et al., 2006; Gómez-Gómez and Boller, 2002). Grapevine VvFLS2, the functional ortholog of AtFLS2, was shown to differentially recognize flg22 from different bacteria and flg22-triggered immune responses was observed to induce partial resistance against *B. cinerea* (Trdá et al., 2014). FLS2 interacts with BAK1 (BRASSINOSTEROID INSENSITIVE ASSOCIATED KINASE 1), an LRR-RLK, forming a complex after elicitation (Chinchilla et al., 2007). BAK1 was also shown to interact with other LRR-RKs after ligand perception, activating downstream signaling responses (Roux et al., 2011; Schulze et al., 2010).

LysM-RLK recognizes oligomers of fungal chitin, a constituent of the fungal cell wall (Miya et al., 2007; Wan et al., 2008). Chitin and chitosan act as PAMPs, eliciting immune responses. In rice, OsCEBiP, a LysM-RLP, was shown to bind to chitin elicitors and to interact with OsCERK1, which contains an intracellular kinase domain, in a chitin-dependent manner (Kaku et al., 2006; Shinya et al., 2012). In Arabidopsis, *AtCERK1/LYK1*, a homolog of *OsCERK1*, was also demonstrated to be involved in chitin signaling (Miya et al., 2007; Wan et al., 2008). In grapevine, *VvLYK1-1* and *VvLYK1-2*, orthologs of *AtCERK1/LYK1* and *OsCERK1*, were reported to functionally complement the *Atcerk1* mutant, restoring chitooligosaccharide-induced immune responses (Brulé et al., 2019). Moreover, VvLYK1-1 played an important role in basal resistance against *E. necator*, restoring the penetration resistance in the *Atcerk1* mutant (Brulé et al., 2019).

Pathogenic species adapted to a specific host are supposed to release effectors to repress PTI. In response, host plants have evolved the ETI as a second layer of resistance. During ETI, PTI-suppressing effector molecules are recognized by specific disease resistance (*R*) genes, direct or indirectly through partner proteins, that, in turn, induces several defense responses that share overlapping pathways with PTI (Jones and Dangl, 2006; Tsuda and Katagiri, 2010). ETI is a stronger version of PTI that often leads to a hypersensitive response (HR) at the infection site, characterized by PCD and local necrosis (Boller and Felix, 2009; Jones and Dangl, 2006). Hypersensitive response prevents nutrient uptake by biotrophic pathogens restricting fungal development (Qiu et al., 2015). Most *R* genes encode NBS-LRR proteins, which are cytoplasmatic proteins with a nuclear binding site (NBS) in front of a series of LRRs.

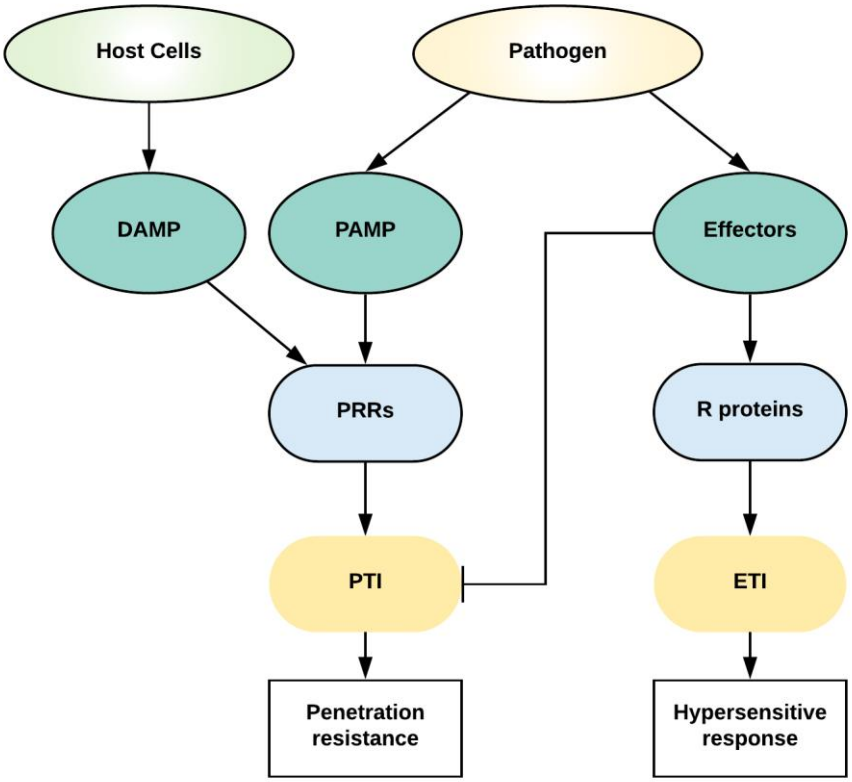

Figure 1.2 – Plant innate immunity system. Plants evolved two primary types of innate immunity: the pathogen-associated molecular patterns (PAMP)-triggered immunity (PTI), and the effector-triggered immunity (ETI). First, plant recognize pathogen-specific molecules (PAMPs) through extracellular pattern-recognition receptors (PRRs), activating the PTI. Affected host cells can also release endogenous damage-associated molecular patterns (DAMPs), acting as PTI elicitors. Effective PTI normally confers penetration resistance. Adapted pathogens release effectors to suppress the PTI. In host cells, effectors are recognized direct or indirectly by specific resistance (R) proteins activating the ETI. ETI is a stronger version of PTI and normally results in a hypersensitive response at the infection site, characterized by programmed cell death and local necrosis.

1.3.1. Host response to Powdery Mildew infection

Different members of the Vitaceae family display a diverse range of responses to PM infection, and resistance is classified into four levels: susceptibility, partial resistance, penetration resistance, and PCD associated resistance (Feechan et al., 2011). Most North American *Vitis* species are resistant to PM, contrary to the Eurasian species, *V. vinifera*.

Although *V. vinifera* cultivars are highly susceptible to powdery mildew, they are able to initiate a basal immunity response but unable to restrict fungal growth and disease progression (Marsh et al., 2010).

1.3.1.1. Resistance to non-adapted powdery mildew pathogens

Penetration resistance is the main process of PTI against non-adapted PM pathogens in most plant species and is conferred by localized cell wall appositions (papillae). Through large-scale genetic screens in Arabidopsis, three *PENETRATION* (*PEN*) genes, *PEN1*, *PEN2*, and *PEN3*, were shown to play major roles in penetration resistance against powdery mildews (Collins et al., 2003; Lipka et al., 2005; Stein et al., 2006). Orthologs of these genes in grapevine might have similar functions against non-adapted PM (Qiu et al., 2015). AtPEN1 is a member of the SNARE family (soluble N-ethylmaleimidesensitive factor attachment protein receptor), which includes proteins that mediate membrane fusion events during endomembrane trafficking (Collins et al., 2003). Following PM challenge, AtPEN1 is re-localized from the plasma membrane to the papilla, possible through the formation of multivesicular bodies and exome secretion (Meyer et al., 2009; Nielsen et al., 2012). AtPEN2 is a myrosinase involved in the biosynthesis of antimicrobial molecules that are delivered to the PM infection site via AtPEN3, which is an ATP-binding cassette (ABC) transporter (Bednarek et al., 2009; Lipka et al., 2005; Underwood and Somerville, 2013). In grapevine, *VvPEN1*, an ortholog of AtPEN1/AtSYP121 and HvROR2, was shown to rescue the *Atpen1* mutant by restoring penetration resistance against the non-adapted *E. necator* and was shown to be accumulated in the papilla formed beneath the penetration peg after PM challenge (Feechan et al., 2013b). Accumulation of AtPEN1 and VvPEN1 under the penetration site is inhibited by the endomembrane trafficking inhibitors brefeldin A and wortmannin (Feechan et al., 2013b; Nielsen et al., 2012).

1.3.1.2. Resistance involving effector-triggered immunity

Resistant grapevines have evolved ETI to powdery mildew. Several North American species are resistant to PM and a number of resistance loci have been identified: *Resistance*

against U. necator1 (*RUN1*) (Barker et al., 2005; Pauquet et al., 2001); *RUN2.1* and *RUN2.2* (Riaz et al., 2011), *Resistance against E. necator2 (REN2)* (Dalbó et al., 2001); *REN3* (Welter et al., 2007); and, *REN5* (Blanc et al., 2012). Some of them were used in breeding programs (Feechan et al., 2015; Li et al., 2013). Successful breeding between wild North American *Muscadinia rotundifolia* and susceptible *V. vinifera* resulted in a resistant hybrid due to the introgression of a single dominant locus *RUN1* (Pauquet et al., 2001). *RUN1* is co-located with a locus for *Resistance to Plasmopora viticola* (downy mildew) (*RPV1*) on chromosome 12 and contains a family of seven putative Toll/interleukin-1 receptor (TIR)-NBS-LRR-type R genes (Feechan et al., 2013a). *RUN1*-mediated resistance is characterized by PCA induction within the penetrated epidermal cells (Dry et al., 2010).

Another two PM resistance loci were identified in two different *M. rotundifolia* cultivars, *RUN2.1* in 'Magnolia' and *RUN2.2* in 'Trayshed', on chromosome 18 (Riaz et al., 2011). *REN5* locus was identified in *M. rotundifolia* 'Regale' and located on the upper part of chromosome 14 (Blanc et al., 2012). *REN5* is supposed to act after penetration delaying and stopping mycelium development (Blanc et al., 2012).

Wild Chinese *Vitis* species are also reported as a source of PM resistance, and some resistance loci have been identified: *REN4* (Ramming et al., 2011), *REN6* and *REN7* (Pap et al., 2016). *REN4* from the *V. romanetii* has been successfully introgressed into *V. vinifera* and shown to segregate as a single dominant *R*-locus (Ramming et al., 2011). *REN4* was firstly reported to confer non-race-specific resistance associated with high levels of penetration resistance and not HR-dependent (Ramming et al., 2011). Further studies revealed that *REN4*-mediated resistance occurs post-penetration involving two different mechanisms: PCD of penetrated epidermal cells, or block nutrient uptake through the encasement of the haustoria by callose (Qiu et al., 2015). *REN6* and *REN7* PM resistance loci were identified in *V. piasezkii* and located on chromosomes 9 and 19, respectively (Pap et al., 2016). Both loci act as post-penetration through induction of PCD; *Ren6* confers complete resistance to PM, whereas *Ren7* confers partial resistance with reduced colony size (Pap et al., 2016). Comparison of the resistance responses of the *REN6*, *REN7*, and *RUN1* loci in the same genetic background revealed that *REN6* higher speed and strength of resistance than *RUN1*, which, in turn, is superior to *REN7* (Pap et al., 2016).

Although *V. vinifera* is susceptible to PM, the *Ren1* locus, which provides partial resistance to PM, was identified in central Asian *V. vinifera* 'Kishmish vatkana' and 'Dzhandzhal kara' (Coleman et al., 2009; Hoffmann et al., 2008). *REN1*-mediated resistance does not prevent *E. necator* penetration into the host cells but represses the subsequent hyphal

development (Hoffmann et al., 2008). The *REN1* locus was mapped to a 1.4 Mbp region on chromosome 13, and the same region on the PN40024 *V. vinifera* reference genome contains an NBS-LRR gene cluster (Coleman et al., 2009). Additional cultivated and wild Central Asian grape accessions with partial resistance were shown to carry a *Ren1*-like local haplotype based on the presence of molecular markers associated with *Ren1* (Riaz et al., 2013). Riaz et al. (2013) also suggested that *V. vinifera* subsp. *sylvestris* might be the progenitor of the *REN1*-like resistance in Central Asian *V. vinifera* accessions.

Most *R* genes encode NBS-LRR proteins that act as intracellular receptors that specifically recognize the effector molecules secreted during infection and initiate ETI. Expression analysis in infected leaves identified 63 *NB-LRR* genes as PM responsive across seven partially PM-resistant and two PM-susceptible *V. vinifera* accessions (Goyal et al., 2019). The expression levels of *NBS-LRR* genes were higher in partially resistant varieties than in susceptible ones, suggesting that the upregulation of some *NBS-LRR* genes might be important in resistance against PM (Goyal et al., 2019). The majority of the PM-responsive *NBS-LRR* genes were located in chromosomes that harbor previously identified loci responsible for PM resistance (Goyal et al., 2019).

Ectopic expression of *VpCN* in Arabidopsis, whose encoded protein contains an NB-ARC domain, was described to induce resistance to Arabidopsis powdery mildew *Golovinomyces cichoracearum*, suggesting a role in PM resistance in grapevine (Wen et al., 2015).

1.3.1.3. Additional defensive mechanisms

In addition to the defense responses involving *R* genes, several other genes were proposed as being involved in PM resistance, including PR proteins and genes involved in defense signal perception and transduction.

ENHANCED DISEASE SUSCEPTIBILITY1 (EDS1) has been reported as an important regulator of plant basal and receptor-triggered immunity. *EDS1* encodes a lipase-like protein, and EDS1 protein interacts with PHYTOALEXIN DEFICIENT4 (PAD4) in the cytoplasm and in the nucleus in distinct complexes and with SENESCENCE ASSOCIATED GENE101 (SAG101) in the nucleus (Feys et al., 2005). Loss of EDS1-PAD4 complexes (in *pad4* mutant) had a negative impact on immune signaling; however, loss of EDS1-SAG101 (in *sag101* mutant plants) was compensated by the presence of EDS1-PAD4 (Feys et al., 2005; Wagner et al., 2013). *EDS1* was shown to be induced in PM-susceptible *V. vinifera* 'Cabernet Sauvignon' upon *E. necator* infection, whereas *EDS1* expression was constitutively high in PM-resistant *V. aestivalis* 'Norton' exceeding the induced levels in 'Cabernet Sauvignon' (Fung et al., 2008).

The level of salicylic acid (SA) was also constitutively higher in 'Norton' than in 'Cabernet Sauvignon' in non-infected conditions (Fung et al., 2008). Four EDS1-LIKE (EDL) genes were identified in *V. vinifera* and were responsive to SA (Gao et al., 2010). Moreover, ectopic expression of both *VaEDS1* and *VvEDS1* in Arabidopsis was able to restore the *eds1* mutant (Gao et al., 2010). *VaEDS1*, *VvEDS1*, and *VaEDL2* were found to confer resistance to PM in Arabidopsis *eds1-1* mutants and promoters of both *VaEDS1* and *VvEDS1* were induced by SA (Gao et al., 2014). EDS1-PAD4 complex is essential for SA-mediated defenses (Rietz et al., 2011).

PR proteins are triggered during pathogen infection and usually possess enzymatic functions. Two PR proteins, chitinase and *β*-1,3-glucanase, were shown to have increased activity in powdery mildew infected grape leaves (Giannakis et al., 1998). Introduction of a rice chitinase gene (*RCC2*) into somatic embryos of grapevine (*V. vinifera* 'Neo Muscut') resulted in enhanced resistance against *E. necator* infection (Yamamoto et al., 2000). *VpPR-10.1* expression from the Chinese wild grapevine *V. pseudoreticulata* was shown to be affected by *E. necator* infection (Xu et al., 2014). Moreover, VpPR-10.1 possesses both RNase and DNase activity and can lead to PCD and DNA degradation; its nuclease activity showed a strong correlation with the antifungal property (Xu et al., 2014). Therefore, VpPR-10.1 was suggested to have a critical role in grapevine resistance, potentially playing a dual role in degrading pathogen RNA and inducing PCD of host tissues (Xu et al., 2014). PR-4 protein (VpPR4-1) from *V. pseudoreticulata* was shown to enhance resistance to PM infection when overexpressed in *V. vinifera* 'Red Globe' (Dai et al., 2016).

Members of the *Really Interesting New Gene (RING)* finger protein gene family from the Chinese wild grapevine *V. pseudoreticulata* Baihe 35-1 accession were found to induce PM resistance when ectopically overexpressed in Arabidopsis (Yu et al., 2013, 2011). E3 ubiquitin ligase *Erysiphe necator*-induced RING finger protein 1 (*EIRP1*) encoded a C3HC4-type RING finger protein with E3 ligase activity and was suggested to positively regulate plant disease resistance by mediating proteolysis of the negative regulator VpWRKY11 via degradation by the 26S proteasome (Yu et al., 2013).

Several WRKY transcription factors were found to be involved in grapevine resistance to PM infection, possible through activation of defense-related genes. *VpWRKY1* and *VpWRKY2* were shown to be induced by *E. necator* infection, and the expression level of *VpWRKY1* was found to be correlated with the level of resistance (Li et al., 2010). Additionally, ectopic expression of *VpWRKY1* or *VpWRKY2* in Arabidopsis enhanced resistance to PM (Li et al., 2010). *VqWRKY52* expression was induced by powdery mildew and SA treatment, but not by

jasmonic acid, and ectopic expression in Arabidopsis induced resistance to powdery mildew and *Pseudomonas syringae* pv. tomato DC3000, however enhanced susceptibility to *Botrytis* infection (Wang et al., 2017). Overexpression of *V. davidii WRKY53* in Arabidopsis was also found to enhance resistance to a wide range of pathogens, including PM *Golovinomyces cichoracearum*, *Coniella diplodiella*, and *P. syringae* (Zhang et al., 2019).

Lignin, phytoalexins, and phenolic compounds also play crucial roles in grapevine defense against pathogens. Stilbenoids are important phytoalexins, and their accumulation has been correlated with enhanced tolerance against PM in grapevine (Belhadj et al., 2006; Schnee et al., 2008). Accumulation of *trans*-resveratrol was found to accumulate in PM infected berries (Piermattei et al., 1999). Stilbene synthase genes, which encode a key enzyme in the stilbene synthesis, were shown to be induced upon *E. necator* infection in susceptible grapevine (Fung et al., 2008). Accumulation of stilbenes was suggested to be restricted to infected cells penetrated by an appressorium–peg (Schnee et al., 2008).

1.3.1.4. Genes involved in Susceptibility towards powdery mildew

An alternative approach to confer resistance to PM is based on susceptibility genes (*S*-genes), which loss-of-function leads to resistance (Kusch and Panstruga, 2017). Successful penetration by adapted PM pathogens has been related to the presence of a functional allele of the *MILDEW RESISTANCE LOCUS O* (*MLO*). Inactivation of specific *MLO* genes was reported to confer broad-spectrum resistance to PM in several host plant species (Bai et al., 2008; Consonni et al., 2006; Humphry et al., 2011; Jørgensen, 1992). MLO proteins are localized in the plasma membrane and contain a seven-transmembrane domain and a C-terminal calmodulin-binding domain (Devoto et al., 1999; Kim et al., 2002). Although the biological function of MLO proteins remains elusive, they are proposed to be involved in vesicle trafficking and to negatively regulate both actin-dependent and -independent defenses against PM pathogens (Miklis et al., 2007; Panstruga, 2005).

In grapevine, three of the 17 putative *VvMLO* genes, *VvMLO3*, *VvMLO4*, and *VvMLO17*, were suggested to have a role in susceptibility to PM, based on the high similarity with *MLO S*-genes from barley, Arabidopsis and tomato, and the transcription levels during PM infection (Feechan et al., 2008; Winterhagen et al., 2008). Moreover, *VvMLO3* and *VvMLO4* were shown to rescue the Arabidopsis *mlo2 mlo6 mlo12* triple mutant, which displays complete immunity to PM (Feechan et al., 2013b). VvPEN1 and VvMLO3/VvMLO4 were shown to share the same endocytic trafficking pathway, suggesting that these proteins are recycled from the plasma membrane to papillae at the site of attempted fungal penetration (Feechan et al., 2013b).

Knockdown of multiple *VvMLO* genes resulted in resistance to PM, and *VvMLO6* and *VvMLO7* were identified as candidates for PM susceptibility in *V. vinifera*, with a possible additive activity (Pessina et al., 2016).

Furthermore, a new PM susceptibility QTL was mapped in *V. vinifera* 'Chardonnay' by genotype-by-sequencing approach and named *Sen1* (*Susceptibility to Erysiphe necator 1*) (Barba et al., 2014).

1.3.2. Hormones in defense

Plant hormones are small molecules that regulate several developmental and physiological processes, including plant defense. SA and jasmonate (JA) are classically recognized as the major players in defense. Nevertheless, ethylene (ET), ABA, auxins, gibberellins, cytokinins, and brassinosteroids have been reported as also involved in defense responses. These phytohormones respond to stress via synergistic and antagonistic actions often referred to as signaling crosstalk (Checker et al., 2018).

Salicylic acid is a phenolic compound derived from the shikimate-phenylpropanoid pathway and has an essential role in basal defenses, ETI, and in systemic acquired resistance. SA has been associated with the activation of defenses against biotrophs and hemibiotrophs, but it also appears to induce susceptibility to necrotrophic pathogens by antagonizing the JA signaling and by inhibition auxin signaling (Blanco-Ulate et al., 2013; Glazebrook, 2005). Pathogen infection induces the accumulation of SA (Berens et al., 2017). Susceptible grapevine accumulates SA in response to *E. necator* infection, but SA levels in resistant grapevine are constitutively high (Fung et al., 2008). Activation of the SA pathway at the site of infection triggers a defense response in distal parts of the plant (Maruri-López et al., 2019).

Jasmonates have been described as a positive regulator of plant immune responses against insects and necrotrophic pathogens (Glazebrook, 2005). However, JA/ET-related defenses have been shown to be effective against adapted PM pathogens when induced constitutively, artificially or systemically (Belhadj et al., 2006; Ellis et al., 2002; Stein et al., 2008). Isoleucine-conjugate of jasmonic acid (JA-Ile) is known as the most active endogenous form of JA (Fonseca et al., 2009; Staswick and Tiryaki, 2004).

Ethylene is a gaseous hormone in higher plants that controls a variety of immune responses in conjunction with other signaling networks. Ethylene has been shown to induce the synthesis of phytoalexins, lignin, and other phenolic compounds as well as the activation of enzymes including chitinases, β-1,3-glucanases, phenylalanine ammonia-lyase (PAL), peroxidase, and other defense-related proteins (Koehl et al., 2007). On the other hand, ET also

promotes senescence or ripening processes that facilitate infection by pathogens (Blanco-Ulate et al., 2013). Additionally, exogenous application of ethephon, an ethylene-releasing substance, increased resistance against *E. necator* infection in grapevine detached leaves and foliar cuttings, through the activation of defense responses (Belhadj et al., 2008).

ABA plays a crucial role in tolerance to abiotic stress; however, it also influences plant-pathogen interactions, acting as an inducer or repressor of plant defense depending on the specific plant-pathogen interaction (Robert-Seilaniantz et al., 2011). ABA was shown to suppress plant resistance by antagonizing SA- and JA/ET-dependent immune responses against disease and herbivore attack (Blanco-Ulate et al., 2013; Sánchez-Vallet et al., 2012). Additionally, many pathogens can directly produce ABA or induce host ABA synthesis to increase susceptibility by accelerating fruit ripening and suppressing SA-mediated defenses (Berens et al., 2017; Siewers et al., 2004). ABA was also suggested to play a role in regulating the carbohydrate supply to tissues under biotic stress (Hayes et al., 2010).

Auxins have been reported as being involved in plant defense (Berens et al., 2017; Kidd et al., 2011). Auxins were suggested to be involved in the grape response to *Botrytis* infection (Agudelo-Romero et al., 2015). Moreover, auxin interacts with JA in resistance to necrotrophic pathogens (Qi et al., 2012). Recently, an accumulation of compounds from the auxin group, except IAA (indole-3-acetic acid), was observed in barley infected with powdery mildew (Janeczko et al., 2019).

1.3.3. Transcription factors in biotic stress responses

Transcription factors (TFs) are central regulators of gene expression, modulating several plant processes, including defense responses. Furthermore, TFs might interact with other TFs or with additional nuclear proteins to modulate gene expression. Several transcription factor families have been linked to plant stress response, and the most studied are WRKY, MYB, NAC, and bZIP. WRKY proteins are unique to plants, and specific members were shown to be induced by several pathogens, defense signals, and wounding (Eulgem, 2005; Eulgem et al., 2000). Several WRKY factors were shown to be negative regulators of plant defense, whereas others positively modulate these responses, creating a complex transcriptional network with positive and negative feedback loops and feed-forward modules (Eulgem and Somssich, 2007). Members of the R2R3-MYB subfamily have been associated with modulation of the phenylpropanoid biosynthetic pathway, which is associated with pathogen defense and resistance (Czemmel et al., 2012; Duan et al., 2016; Höll et al., 2013). A NAC member was shown to be induced by powdery mildew infection in grapevine in a SA-independent manner

(Toth et al., 2016). Specific members of the bZIP family are involved in the regulation of stress resistance (Kaminaka et al., 2006; Kuhlmann et al., 2003; Meng et al., 2005; Zhang et al., 2008). Overexpression of grapevine VvbZIP60 in Arabidopsis was shown to increase resistance to powdery mildew through the salicylic acid signaling pathway (Yu et al., 2019).

Other transcription factor families that have been more recently associated with biotic stress response are the LATERAL ORGAN BOUNDARIES domain (LBD) and the GRAS families.

1.3.3.1. LATERAL ORGAN BOUNDARIES (LOB) Domain (LBD) transcription factor family

LATERAL ORGAN BOUNDARIES (LOB) Domain (LBD) transcription factor family is characterized by a conserved LOB domain at the N-terminal. Members of this family have crucial roles in the regulation of plant organ development (Majer and Hochholdinge, 2011; Xu et al., 2016).

Specific *LBD* genes are also involved in disease susceptibility. *AtLBD20*, a root-specific gene, was the first *LBD* genes associated with susceptibility, acting as a susceptibility gene to *Fusarium oxysporum* (Thatcher et al., 2012b). Loss-of function mutation of the *AtLBD20* gene led to enhanced resistance to *F. oxysporum*, while overexpression resulted in higher susceptibility after infection (Thatcher et al., 2012b). Moreover, AtLBD20 was suggested to have a role in JA signaling, repressing the expression of the JA-regulated defense genes *THIONIN2.1* (*THI2.1*) and *VEGETATIVE STORAGE PROTEIN2* (*VSP2*). (Thatcher et al., 2012b). Analysis of gene expression of some *AtLBD* genes, particularly from Class II, was shown to be induced by multiple pathogens indicating a role in plant defense responses (Thatcher et al., 2012a). *AtLBD16*, which is involved in auxin-triggered lateral-root development, has a function on gall and giant cells formation, that comprise the feeding structures of root-knot nematodes (RKN) (Cabrera et al., 2014). *Citrus sinensis CsLOB1* was found to be a susceptible gene in citrus bacterial canker, caused by multiple *Xanthomonas* species (Hu et al., 2014). Some of the type II secretion (T3S) transcription activator-like (TAL) effectors secreted by bacterial pathogens induces the expression of *CsLOB1* by TAL binding to the promoter region, inducing pustule formation and enhance bacterial growth (Hu et al., 2014). Moreover, *CsLOB2* and *CsLOB3*, which are *CsLOB1* homologs, were found to have a similar role as *CsLOB1* in citrus bacterial canker (Zhang et al., 2017).

1.3.3.2. GRAS transcription factor family

GAI (gibberellic acid insensitive), RGA (repressor of GA1) and SCR (scarecrow) were the first three functionally characterized GRAS genes and named this protein family (Di Laurenzio et al., 1996; Peng et al., 1997; Pysh et al., 1999; Silverstone et al., 1998). GRAS genes are proposed to encode transcription factors since several family members were located in the nucleus and showed transcriptional activation capabilities (Itoh et al., 2002; Morohashi et al., 2003; Silverstone et al., 1998). GRAS proteins present high sequence homology to each other in the C-terminus, where five conserved motifs can be found. By contrast, the N-terminal is highly variable, both in sequence and length, suggesting that this region might be responsible for the functional specificity (Tian et al., 2004). Furthermore, a subgroup of GRAS proteins named DELLA proteins act as repressors of gibberellin signaling and share a DELLA motif in the N-terminal region (Silverstone et al., 1998).

Several GRAS proteins have been functionally characterized in diverse plant species and were reported to be involved in several biological processes (Bolle, 2016). Although little is known about the involvement of GRAS proteins in plant defense, some studies reported that some GRAS genes are responsive to biotic stress. Six tomato *SlGRAS* transcripts were shown to be accumulated during the onset of disease resistance to *Pseudomonas syringae* pv. *tomato* and to be induced in response to mechanical stress (Mayrose et al., 2006). Accumulation of *SlGRAS* transcripts upon mechanical stress was, in part, dependent on jasmonic acid. Additionally, suppression of *SlGRAS6* impaired the resistance to *P. syringae* (Mayrose et al., 2006). In tobacco leaves, *NtGRAS1* expression was strongly induced after antimycin A (an inhibitor of mitochondrial electron transport) treatment or upon *P. syringae* infection (Czikkel and Maxwell, 2007). Two GRAS genes from rice, *CIGR1* and *CIGR2*, were induced by the fungal elicitor N-acetylchitooligosaccharide and suggested to act as transcriptional regulators in the elicitor-induced defense response in rice (Day et al., 2003). Arabidopsis SCL14 interacts with class II TGA transcription factors, acting as a transcriptional coactivator of stress-inducible promoters, especially SA- and 2,4-D-inducible promoters (Fode et al., 2008). The TGA/SCL14 complex was therefore suggested to be involved in the activation of a general broad-spectrum detoxification network when challenged by xenobiotics (Fode et al., 2008). DELLA proteins were reported to promote susceptibility to virulent biotrophs and resistance to necrotrophs by modulating the balance of salicylic and jasmonic acid signaling pathways (Navarro et al., 2008) In grapevine, several GRAS genes were found to be up-regulated upon biotic stress challenges, such as *VviRGA5*, *VviLISCL12*, *VviLISCL3*, *VviPAT3*, *VviPAT4*, and *VviHAM3* (Grimplet et al., 2017).

1.4. Targeted genome editing using CRISPR-Cas9: applications in fruit quality and stress resilience

Plant domestication was essential in the transition from hunting-gathering behavior to agriculture in the Neolithic period (Gepts, 2014; Purugganan and Fuller, 2009). Starting with trait selection then moving to conventional breeding, several plant crops were modified to increase productivity, nutritional value, improve quality traits like flavor, color, and juiciness, and gain resistance to stress conditions. With the development of genetic engineering, New Plant Breeding Techniques (NPBTs) were developed and are currently being applied. Genomic studies have shown that targeted DNA double-strand breaks (DSBs) by engineered nucleases lead to genome editing or transgene integration through homologous recombination (Gascuel et al., 2017). At the cellular level, there are two DNA break repair pathways that drive to different genome modifications: non-homologous end joining (NHEJ) and homology direct repair (HDR). In blunt-end DSB, NHEJ repair can lead to random insertions or deletion (indels), which can cause frameshift mutation of a coding sequence or disruption of the *cis*-acting element in promoters or enhancers. When an exogenous DNA template homologous to the surrounding sequence of the DSB is supplied, HDR is used to introduce precise point mutation or specific nucleotide sequences. The most used technologies for targeted DSB were zinc finger nucleases (ZFN; Kim et al., 1996) and transcription activator-like effector nucleases (TALENs; Christian et al., 2010). Both are engineered fusion proteins with a DNA-binding domain fused with a nonspecific nuclease domain. Most recently, a new platform is gaining ground based on archaea and bacterial adaptive immunity system: the type II Clustered Regulatory Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated protein 9 nuclease (Cas9) (reviewed by Bortesi and Fischer, 2015; Sander and Joung, 2014). CRISPR was firstly identified in 1987 (Ishino et al., 1987) and applied to genome editing in 2013 (Cho et al., 2013; Cong et al., 2013; Mali et al., 2013; Hwang et al., 2013).

The CRISPR is a protective system against invading nucleic acids, like viruses and plasmids, acting by cleaving the foreign DNA in a sequence-dependent manner. Type II CRISPR systems incorporate fragments of the foreign DNA (known as spacers) between the CRISPR repeats as an array within the bacterial host genome. During infection, these arrays are transcribed and processed into small interfering CRISPR RNAs (crRNAs) combined with a transactivating CRISPR RNA (tracrRNA) in order to activate and guide the Cas9 nuclease to the homologous sequence in the foreign DNA (known as protospacers). The presence of a conserved three nucleotides protospacer-adjacent motif (PAM) is mandatory for cleavage; the

most common is the 5'-NGG-3' motif. The twelve nucleotides upstream of the PAM are responsible for the specificity of the tracrRNA to the targeted DNA sequence.

The transition from the CRISPR system of *Streptococcus pyogenes* to the targeted genome editing occurred when Jinek et al. (2012) found that the target DNA could be modified by changing the crRNA and that this could be fused with the tracrRNA into a single guide RNA (sgRNA). The CRISPR-Cas9 system became the simplest technology: (1) two components, Cas9 nuclease and a gRNA, must be expressed or introduced in target cell of organism, (2) the twenty bases at the 5' end of the sgRNA guide the Cas9 to the target DNA by RNA-DNA complementarity, and (3) the target site must be immediately upstream of a PAM sequence, normally a 5'-NGG motif.

CRISPR-Cas9 technology has been showing promising results in fruit crop improvement that will be discussed in the following sections (Table 1.1). These studies were mainly applied in tomato, citrus, and cucumber. Nevertheless, the applicability of CRISPR-Cas9 in gene editing was successful in several other fruit crops like grapevine (Malnoy et al., 2016; Osakabe et al., 2018; Ren et al., 2016), apple (Malnoy et al., 2016; Nishitani et al., 2016), watermelon (Tian et al., 2017), wild and cultivated strawberry (Martín-Pizarro et al., 2019; Zhou et al., 2018), kiwifruit (Wang et al., 2018), and banana (Kaur et al., 2018).

Legislation about plants edited with the CRISPR-Cas9 system is still on debate. In the United States, if there is no trace of any foreign genetic material, the United States Department of Agriculture (USDA) does not consider as GMOs (genetically modified organisms) the organisms generated by targeted mutagenesis with self-repair mechanisms (Jones, 2015; Ledford, 2013; Waltz, 2012). On the other hand, in European Union plants created with CRISPR or other gene-editing tools are considered as GMOs and not derived from conventional mutagenesis; therefore, they will follow very stringent rules to be cultivated (Callaway, 2018; Kupferschmidt, 2018). CRISPR-Cas9 system has been applied integrating the Cas9 gene and the sgRNA into the genome of the edited organism at the initial stage; however, studies demonstrated that through segregation is possible to obtain homozygous mutated plants without the transgenic part in the second or third generation. These final homozygous lines should not be considered as transgenic plants since the final organism has no difference compared to classic mutagenesis such as caused by irradiation. Still, using this strategy the T0 generation integrates transgenic genes, which can raise legal problems. To overcome this problem some studies have been focused on exploring the direct delivery of purified sgRNA-Cas9 ribonucleoproteins (RNPs) into the target tissue/cells. Malnoy et al. (2016) successfully edited both grape and apple protoplasts using RNPs, avoiding the integration of plasmid DNA into the

target genome. They targeted the *MLO-7* gene in grapevine and *DIPM-1*, *DIPM-2*, and *DIPM-4* in apple in order to increase resistance against powdery mildew and fire blight disease, respectively (Malnoy et al., 2016). Optimization of regeneration protocols from edited protoplasts is crucial to apply the RNPs delivery to the development of edited but non-transgenic fruit crop plants.

In these sections, studies conducted so far using CRISPR technology applied to fruit crop quality enhancement and stress mitigation are reviewed.

Table 1.1 – Application of CRISPR-Cas9 genome editing system in fruit crop plants for biotic stress resistance, quality traits, and *de novo* domestication.

Plant	**Factor**	**Target gene**	**Trait**	**Reference**
Citrus (Wanjincheng orange)	Biotic stress	*CsLOB1*	Citrus canker	(Peng et al., 2017)
Citrus (Duncan grapefruit)	Biotic stress	*CsLOB1*	Citrus canker	(Jia et al., 2017)
Cucumber	Biotic stress	*eIF4E*	Virus pathogen	(Chandrasekaran et al., 2016)
Grape	Biotic stress	*VvWRKY52*	*Botrytis cinerea* resistance	(Wang et al., 2016)
Tomato	Biotic stress	*SlMlo1*	Powdery mildew	(Nekrasov et al., 2017)
Tomato	Biotic stress	*SlDMR6*	Pseudomonas syringae, Phytophthora capsici, and Xanthomonas spp	(Thomazella et al., 2016 [Preprint])
Tomato	Quality trait	*PECTATE LYASE (PL)*	Fruit firmness	(Uluisik et al., 2016)
Tomato	Quality trait	*SELF-PRUNING 5G (SP5G) and SELF-PRUNING (SP)*	Photoperiodic response	(Soyk et al., 2017b)
Tomato	Quality trait	*LONG-NON CODING RNA (lncRNA1459)*	Fruit ripening	(Ran Li et al., 2018)
Tomato	Quality trait	*ORGANELLE RECOGNITION MOTIF (SlORRM4)*	Mitochondrial function	(Yang et al., 2017)
Tomato	Quality trait	*SlAGAMOUS-LIKE 6 (SLAGL6)*	Parthenocarpic fruits	(Klap et al., 2017)
Tomato	Quality trait	*SlIAA9*	Parthenocarpic fruits	(Ueta et al., 2017)
Tomato	Quality trait	*ALC* allele	Shelf life	(Yu et al., 2017)
Tomato	Quality trait	*SlGAD2* and *SlGAD3*	GABA accumulation	(Nonaka et al., 2017)
Tomato	Quality trait	*GABA-TP1, GABA-TP2, GABA-TP3, CAT9* and *SSADH*	GABA accumulation	(Rui Li et al., 2018)
Tomato	Quality trait	*SGR1, LCY-E, Blc, LCY-B1,* and *LCY-B2*	Lycopene accumulation	(Xindi Li et al., 2018)
Tomato	Quality trait	*cis*-regulatory elements	Fruit size, inflorescence branching, and plant architecture	(Rodríguez-Leal et al., 2017)
Tomato	Quality trait	*pectate lyase 56 (PL), polygalacturonase 2a (PG2a)* and *β-galactanase (TBG4)*	Pectin degradation in ripening	(Wang et al., 2019)
Wild tomato	*De novo* domestication	*SELF-PRUNING (SP), OVATE (O), FRUIT WEIGHT 2.2 (FW2.2),*	*Solanum pimpinellifolium* domestication	(Zsögön et al., 2018)

		LYCOPENE BETA CYCLASE (CycB), FASCIATED (FAS) and *MULTIFLORA (MULT)*		
Wild tomato	*De novo* domestication	*SP*, *SP5G*, *SlCLV3*, and *SlWUS*	*Solanum pimpinellifolium* domestication	(Tingdong Li et al., 2018)
Groundcherry	*De novo* domestication	*SP*, *SP5G, and CLV1*	*Physalis pruinose* domestication	(Lemmon et al., 2018)

1.4.1. Improvement of traits associated with fruit quality

Improvement of fruit quality is one of the main purposes of the NPBTs. In tomato, several studies were performed using CRISPR-Cas9 system to obtain parthenocarpic tomato fruits (Klap et al., 2017; Ueta et al., 2017), and to increase shelf life (Yu et al., 2017) and fruit yield (Soyk et al., 2017b). Several tomato traits were also reviewed by Rothan and co-workers (2019) and suggested as future targets for tomato improvement.

One of the most important traits for agriculture nowadays is the production of seedless fruits by parthenocarpy, i.e., fertilization-independent fruit set. Breeding for parthenocarpy brings several advantages like stable and sustainable crop production in the context of climate changes and higher content of total soluble solids (Klap et al., 2017). Moreover, seedless fruits are more appreciated by the consumers and more useful in some industries (such as sauce production). Klap et al. (2017) used the CRISPR-Cas9 technology to target loss of function mutation of *SlAGAMOUS-LIKE 6 (SLAGL6)* gene. The MADS-box gene *SLAGL6* was suggested to be involved in the regulation of "ovary arrest" until fertilization and its mutation conferred facultative parthenocarpy. Under heat stress, mutants are capable of seedless fruit production with similar characteristics to wild type (WT). As the pollen viability and sexual reproduction are maintained, this study suggests the use of *SLAGL6* for facultative parthenocarpy (Klap et al., 2017). Furthermore, Ueta et al. (2017) also suggest a rapid breeding strategy to obtain seedless tomato fruits using CRISPR-Cas9. *SlIAA9*, a key regulator of auxin signaling involved in repression of fruit initiation without fertilization, was mutated with very high-efficiency rates in both Micro-Tom and Ailsa Craig cultivars. Bi-allelic and homozygous mutated T0 plants shown typical parthenocarpy phenotypes. In some cases, fertilized fruits were developed with few seeds that inherited the mutation and exhibited the seedless phenotype (Ueta et al., 2017). These studies proved the efficiency of the CRISPR-Cas9 system in improving tomato quality traits.

Another critical trait of fleshly fruits for farmers is the shelf-life, and one of the main objectives of the breeding programs is to extend this period. In tomato plants, several natural mutations slow down ripening conferring longer shelf life such as *rin* (ripening inhibitor), *nor* (non-ripening) and *alc* (*alcobaca* syn. *DFD*, delayed fruit deterioration). The most used strategy

so far to increase shelf life is the hybridization with mutated varieties, which is time-consuming and may compromise other quality traits like flavor and color. With CRISPR-Cas9 it will be possible to rapidly replace the target locus in order to extend the storage time. In fact, HDR-mediated replacement of *ALC* allele for the recessive *alc* in M82 tomato fruits resulted in a longer shelf life (Yu et al., 2017). In this work, the homozygous *alc* line without the T-DNA insertion was generated at T1 generation, and it showed better performance during storage than WT (Yu et al., 2017). Prevent fruit softening is another strategy to increase shelf life. Most fruits undergo softening during ripening, which is important for fruit quality but a problem for storage and transportation. Pectin degradation on fruit cell walls is on the basis of fruit softening during ripening (Brummell, 2006). *Pectate lyase* (*PL*) was reported as critical in fruit softening, and its targeted mutation affects firmness without disturbing ripening aspects like ethylene biosynthesis, color, total soluble solids, yield or weight (Uluisik et al., 2016). Additionally, Wang et al. (2019) used targeted loss-of-function mutation of genes associated with pectin degradation in tomato (*PL*; *polygalacturonase 2a*, *PG2aI;* and *β-galactanase*, *TBG4*). They found that only the *PL* mutation has a significant impact on fruit softening, although mutations in *PG2a* and *TBG4* influenced fruit color and weight.

RNA editing plays a key posttranscriptional role in gene expression, and its involvement in fruit ripening is being studied. ORRM4 is a mitochondrial RNA editing factor and silencing of *SlORRM4* gene was shown to delay tomato fruit ripening by lowering the respiratory rate and ethylene production and was localized to the mitochondria (Yang et al., 2017). SlORRM4 mutation disrupted RNA editing function for several mitochondrial genes. This study has shown the involvement of RNA editing factors in fruit ripening (Yang et al., 2017).

With the increased concern about human health and life quality, nutraceutical properties are desirable for the prevention of some health problems. The γ-aminobutyric acid (GABA) is a non-protein amino acid found in bacteria, animals, and plants. Several studies have been shown to confer health benefits like lowering blood pressure of hypertensive patients, and the consumption of GABA-enriched foods is being promoted (Takahashi et al., 1961, 1955; Yoshimura et al., 2010). An efficient increase of GABA content was obtained in tomato fruits (Nonaka et al., 2017). GABA is synthesized from glutamate by the glutamate decarboxylase (GAD), which generally has an autoinhibitory domain at the C-terminus. Nonaka et al. (2017) used target mutagenesis to knock-out the autoinhibitory part of two tomato GAD genes (*SlGAD2* and *SlGAD3*), deletion of C-terminus shown increased GABA accumulation in comparison with WT. In particular, SLGAD3 with truncated C-terminal presented increased GABA content without significantly disturbing the plant size, flowering, and fruit yield,

suggesting that *SLGAD3* would be a more suitable target for future breeding using targeted mutation (Nonaka et al., 2017). Another study also increased the GABA content in tomato fruits using a multiplexed pYLCRISPR/Cas9 system (Rui Li et al., 2018). Five key genes of GABA metabolism (*GABA-TP1, GABA-TP2, GABA-TP3, CAT9,* and *SSADH*) were targeted using a single vector. Despite the lack of efficiency of one sgRNA, it was possible to obtain a quadrupole mutant proving the applicability of multiplex CRISPR-Cas9 system in tomato genome editing (Rui Li et al., 2018). Further studies will be essential to improve sgRNA efficiency.

Another bioactive compound associated with preventing health problems is lycopene, a C_{40} carotenoid synthesized during fruit ripening. In ripe tomato, lycopene is the most abundant carotenoid and confers color and fruit quality. Recently, Xindi Li and co-workers (2018) used a bidirectional strategy to promote lycopene biosynthesis and prevent its catabolism. Five target genes were selected (*SGR1* to promote lycopene synthesis; and, *LCY-E, Blc*, *LCY-B1*, and *LCY-B2* to prevent lycopene cyclization) and six sgRNA were assembled in the pYLCRISPR/Cas9 vector (Xindi Li et al., 2018; Ma et al., 2015). Single to quadruple mutants were obtained, and they showed higher lycopene and *β*-carotene content than WT plants. The single *SlSGR1* mutant presented the higher lycopene content, about 5.1-fold in comparison to WT, and a more vivid color after the breaker stage (Xindi Li et al., 2018). The mutation was transmitted to subsequent generations (Xindi Li et al., 2018).

Flowering and inflorescence architectures are also important for fruit production. Seasonal changes in day length trigger flowering and the photoperiod response can limit the geographical range of plant cultivation. Soyk et al. (2017b) have shown that genome editing of the tomato flowering repressor, *SELF-PRUNING 5G* (*SP5G*), improves inflorescence architecture and rapid flowering that lead to early fruit yield. The same authors used the CRISPR-Cas9 system to study inflorescence architecture and branched varieties (Soyk et al., 2017a). They identified two mutations in two closely related MADS-box transcription factor genes (*j2* and *ej2*) and verified a dosage relationship among natural and gene-edited mutations that could lead to improved inflorescence architecture and yield (Soyk et al., 2017a).

Alternatively, to target CDS regions, Rodríguez-Leal et al. (2017) targeted *cis*-regulatory elements (CREs) in promoters, which resulted in several novel *cis*-regulatory alleles for three tomato genes that regulate fruit size, inflorescence branching, and plant architecture. Naturally occurring CRE mutations lead to modification of gene expression and have contributed to domestication (Meyer and Purugganan, 2013). This study opens a new window in the genome-editing strategies: CREs are now a potential target to enhance variability for quality traits and

more studies are needed to understand the involvement of the several functional motifs that are identified in the promoter regions.

1.4.2. Mitigation of climate change effects on agricultural productivity

Improvement of crop yield and quality is the aim of many breeders. In the present scenario, the concern about sustainable agriculture, climate changes, and overpopulation are growing. Climate changes are one of the main topics in international political debates and researchers are putting their effort in order to mitigate its effect.

Conventional breeding is being used for introgression of stress-tolerance genes from wild species into cultivated crops; however, this strategy is time-consuming. In several crops, wild relatives are found to be adapted to challenging environments, therefore, *de novo* domestication of wild species using target genome editing is being proposed (reviewed by Khan et al., 2019; Tingdong Li et al., 2018; Østerberg et al., 2017; Zsögön et al., 2018, 2017). Many of the domestication traits are monogenic and have Mendelian inheritance patterns (Doebley et al., 2006; Meyer and Purugganan, 2013). In a review presented by Zsögön et al. (2017), several genes affecting productivity, fruit quality and stress resilience in tomato are discussed and suggested as targets for *de novo* domestication. The same authors designed two separate CRISPR-Cas9 strategies in order to domesticate the wild species *Solanum pimpinellifolium* by targeting the coding region of four genes important for key traits: general plant growth habit, fruit shape and size, fruit number, and nutritional quality (Zsögön et al., 2018). Studies indicate that semi-determinate growth habit combines advantages of the determinate and indeterminate growth, like increased yield, Brix and water-use efficiency (Vicente et al., 2015). Edited *S. pimpinellifolium* lines presented higher fruit size and number than WT and showed a higher fruit lycopene accumulation in comparison with *S. lycopersicum* cv. MicroTom. In a similar study, Tingdong Li et al. (2018) also intent to domesticate the *S. pimpinellifolium* targeting not only coding regions but also promoter region of genes related with day-length sensitivity (*SELF-PRUNING 5G*, *SP5G*), shoot architecture (*SELF PRUNING*, *SP*), flower and fruit production (*CLAVATA3, CLV3,* and *WUSCHEL*, *WUS*), and nutrient content (*GGP1*, vitamin C–biosynthetic enzyme). These studies give rise to a new level of domestication of wild species to overcome challenging environmental conditions without compromising quality traits that are normally affected in traditional breeding, such as flavor and nutritional properties. Additionally, Lemmon et al. (2018) used CRISPR-Cas9 to perform *de novo* domestication of the orphan *Solanaceae* crop 'groundcherry' (*Physalis pruinosa*) editing genes associated with productivity traits such as plant architecture, flower production, and fruit size.

Besides adaptation to climate change, plants are expected to face increased susceptibility to several pathogens. Susceptibility (*S*) genes are important in plant-pathogen interaction and mutation of those genes can lead to higher disease resistance. An example is the *eukaryotic translation initiation factor 4E* (*eIF4E*), a plant translation factor crucial for the cellular infection cycle of potyviruses. In cucumber, Chandrasekaran et al. (2016) applied the CRISPR-Cas9 technology to increase virus recessive resistance through mutation of the *eIF4E*. Cucumber genome has one copy of each *eIF4E* and *eIF(iso)4E*. In this study, targeted mutagenesis with specific sgRNAs targeting nonhomologous regions of exons 1 and 3 of *eIF4E* generated small deletions in the targeted sites. Homozygous T3 cucumber plant lines showed higher resistance to three important viruses, namely *Cucumber vein yellowing virus* (Ipomovirus) and potyviruses *Zucchini yellow mosaic virus* and *Papaya ring spot mosaic virus*-W. Moreover, three backcrossings were sufficient to lose the Cas9 gene producing non-transgenic plants (Chandrasekaran et al., 2016). Higher resistance to potyviruses was also obtained in Arabidopsis disrupting the same gene (Pyott et al., 2016).

The *CsLOB1* was shown to be a critical *S* gene for citrus canker, caused by *Xanthomonas citri* ssp. *citri* (Xcc). It promotes pathogen growth and erumpent pustule formation (Hu et al., 2014). Duncan grapefruit (*Citrus x paradisi*) contains two alleles of the *CsLOB1* and CRISPR-Cas9 was applied to mutate the coding region of the *CsLOB1* in both alleles leading to citrus canker resistant plants (Jia et al., 2017). Moreover, *CsLOB1* contains an effector-binding element (EBE_{PthA4}) which is recognized by the PthA4 effector of Xcc and activates *CsLOB1* expression. Another strategy to increase citrus canker resistance was implemented by Peng et al. (2017); sgRNA was designed to target the EBE_{PthA4} effector-binding element in the promoter of *CsLOB1* in *Citrus sinensis* var. Wanjincheng. Deletion of the entire EBEPthA4 sequence from both *CsLOB1* alleles provided increased resistance to Xcc. Edition of the *CsLOB1* promoter region alone was sufficient to enhance citrus canker resistance in Wanjincheng orange (Peng et al., 2017). In this context, genome-wide analysis of the *LOB domain* gene family in grapevine enabled the identification of potential targets for increasing stress resilience in this important fruit crop (Grimplet et al., 2017). Additionally, promoter analysis revealed several motifs associated with stress responses that could be targeted and studied (Grimplet et al., 2017).

Another important *S* gene that shows a high impact in powdery mildew susceptibility is the *MILDEW RESISTANT LOCUS O* (*Mlo*) which encodes a membrane-associated protein conserved in monocots and dicots (Devoto et al., 1999). In tomato, the *SlMlo* coding sequence was targeted with two sgRNAs separated for 48 bp leading to a deletion of that size and to full

resistance to powdery mildew fungus *Oidium neolycopersici* (Nekrasov et al., 2017). From this study, a non-transgenic plant was created and named Tomelo variety and authors suggest that the *slmlo1* mutation could be easily introduced into elite or locally adapted varieties (Nekrasov et al., 2017).

DOWNY MILDEW RESISTANCE 6 (*DMR6*) has also been studied to increase broad-spectrum resistance against multiple pathogens. Two *AtDMR6* Arabidopsis orthologs were identified in tomato (*SlDMR6–1* and *SlDMR6–2*), and *SlDMR6–1* is up-regulated during infection with *Pseudomonas syringae* pv. *tomato* and *Phytophthora capsici* (Thomazella et al., 2016 [Preprint]). In this work, disruption of exons 2 and 3 using CRISPR-Cas9 resulted in truncated versions of the DMR6. Homozygous T1 line showed resistance to several pathogens, namely the bacteria *Xanthomonas gardneri* Xg153, *Xanthomonas perforans* Xp4b, and *P. syringae* DC3000 and the oomycete *P. capsici* LT1534, without affecting the plant development and morphology (Thomazella et al., 2016 [Preprint]). The authors refer that they are evaluating the performance of these tomato varieties under conditions that are relevant to agricultural practices (Thomazella et al., 2016 [Preprint]).

One fruit crop that is suffering with climate change is the grapevine. Grapevine is a very important crop with a high impact on the global economy, in particular in the Mediterranean countries. Grapevine is very susceptible to several pathogens and a lot of effort is being done to increase resistance by breeding with other resistant *Vitis* species. The main problem with these procedures is the decrease of organoleptic properties of grapes and, consequently, of wine. Genome editing could be applied to induce resistance to several pathogens in *V. vinifera* species. Preliminary studies have been performed on grapevine cell cultures to optimize CRISPR-Cas9 technology (Malnoy et al., 2016; Nakajima et al., 2017; Osakabe et al., 2018; Ren et al., 2016) and increase resistance (Wang et al., 2018). The first applications of CRISPR-Cas9 gene editing was performed in 2016 when Ren and co-workers (2016) were able to efficiently mutate the *L-idonate dehydrogenase* (*IdnDH*), responsible for the accumulation of tartaric acid, in 'Chardonnay' suspension cells and to regenerate grape plantlets, and Malnoy et al. (2016) mutated the *MLO-7* gene in grape protoplasts (referred in the introduction section). In the same year, Wang et al. (2016) identified potential CRISPR-Cas9 target sites for grapevine genome editing. More recently, targeted mutagenesis of the *VvWRKY52* gene resulted in increased resistance to fungal infection with *Botrytis cinerea* (Wang et al., 2018). Interestingly, recent hormonome analysis of Trincadeira and Syrah cultivars upon infection with *Botrytis cinerea* have shown that higher basal levels of salicylic acid, jasmonates, and IAA on Syrah cultivar at an early stage of ripening could confer more tolerance to *Botrytis* infection (Coelho

et al., 2019). Therefore, targeting the basal content of key hormones by editing genes associated with hormonal metabolism could be another direction in the improvement of stress tolerance.

The use of CRISPR-Cas9 technology in plant domestication and stress mitigations has been showing promising results and application in other fruit crops is expected.

1.4.3. Computational Analysis

Nowadays the number of non-model plants with their genome sequenced is increasing, which gives a huge advantage for the application of targeted NBTs, in particular of CRISPR technology. With all genome sequenced, the prediction of off-target effect in the rest of the genome is facilitated and several platforms are being developed to identify single guides RNA with more accurate quality scores (reviewed by Hahn and Nekrasov, 2018). One of the platforms available to off-targets prediction is the Cas-OFFinder which has more than ninety plant genomes in its database, including tomato, grapevine, and apple, and has the possibility of requesting the upload of more organism, becoming a very useful tool in CRISPR-Cas9 application (Bae et al., 2014). It also allows the selection of the nuclease used in order to maximize the accuracy of the off-target prediction.

1.4.4. Technology Advancement

The CRISPR (Clustered Regularly Interspaced Palindromic Repeats) refers to tandem repeats flanked by non-repetitive DNA and was firstly described in 1987 in *Escherichia coli* (Ishino et al., 1987) and applied to genome editing in 2013 and since then its application is growing fast. The most used application of CRISPR is the targeted loss-of-function. However, this technology can be applied in the regulation of gene expression using a catalytically inactive Cas9 (dCas9), i.e., dCas9 can specifically bind to the target sequence guided by sgRNA but cannot break the genome. In fact, Piatek et al. (2015) successfully modified the CRISPR-Cas9 platform to modulate gene expression in plants by developing chimeric dCas9-based transcriptional activators and repressors. Moreover, CRISPR-dCas9 was reported to allow epigenetic modifications such as acetylation and methylation of the genome (Thakore et al., 2015). Modification of Cas9 improves the biotechnological application of CRISPR technology. Several additional CRISPR modified systems are being created (reviewed by Khatodia et al., 2016; Schindele et al., 2018). CRISPR-Cpf1 system has been developed as an alternative to the CRISPR-Cas9 system. Cpf1, also known as Cas12a, belongs to the Class II CRISPR system and is also an RNA guided nuclease that makes DSB with alternative noncanonical PAMs, increasing the number of possible DSB targets. Cpf1 recognizes T-rich PAMs, that are located

upstream of the guide RNA, and generates staggered DSBs distal from the PAM. Additionally, Cas13 (also known as C2c2) has recently been reported to target and cleave specific strands of RNA and applied to degrade mRNAs and combat viral RNA replication (reviewed by Schindele et al., 2018). CRISPR-Cas9 has shown high specificity to the target sequence; however, off-target mutations are always a possibility. Dimeric RNA-guided FokI nucleases (RFNs), where Cas9 is replaced by a FokI nuclease, were developed to prevent off-target mutations since RFN cleavage activity depends on two sgRNAs with a specific spacing and orientation (Tsai et al., 2014). All these alternatives of the CRISPR system are creating a new range of possibilities for genome editing and future studies are required to improve their applicability.

1.5. Objectives

Powdery mildew has a high impact on viniculture, affecting the green tissues of the grapevine plants. Although several studies were performed in leaves to understand the resistance mechanisms, little is known about grape berry response to *E. necator* infection. Therefore, the main aim of Chapter 2 was to study the response mechanisms of grape berries to powdery mildew infection in the early stages of fruit ripening. Metabolic and transcriptional profiling of grape berries infected and non-infected with powdery mildew at green (EL33) and *véraison* (EL35) stages were performed. Another objective of this thesis was to comprehend the involvement of plant hormones in powdery mildew response.

Transcription factors are important regulators of several plant physiological and development processes, as well as in defense. *LATERAL ORGAN BOUNDARIES Domain* (*LBD*) transcription factor family was reported to be involved in the regulation of plant organ development and disease susceptibility. The results from Chapter 2 confirmed this assumption. The aim of Chapter 3 was to identify and map *LBD* genes in the grapevine genome, and to predict molecular functions based on the identification of *cis*-acting regulatory elements in promoter regions and on expression data obtained from public databases.

GRAS transcription factors have been suggested to have a role in fruit ripening and defense. Tomato *GRAS10* and its grapevine ortholog *PAT6* were previously identified as putative ripening regulators in both climacteric and non-climacteric fruits. Therefore, the main objective of Chapter 4 was to develop *slgras10* mutant plants for further functional characterization thought target loss-of-function mutation using CRISPR-Cas9 technology. Two guideRNAs were designed and cloned along with the Cas9 and the resistance gene for selection, and tomato stable transformation mediated by *Agrobacterium tumefaciens* was performed. Moreover, overexpression constructs were developed to complement the study.

Finally, in Chapter 5 the main conclusions of each chapter were integrated and discussed in terms of its implications in grapevine biotic stress response and future work, as well as in the context of grapevine improvement.

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Chapter 2

Transcriptional and metabolic regulation of grape berry metabolism upon powdery mildew infection

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The data present in this chapter is under manuscript preparation.

Pimentel, D., Amaro, R., Reichelt, M., Erban, A., Mauri, N., Soares, F., Rego, C., Martínez-Zapater, J.M., Mithöfer, A., Kopka, J., Fortes, A.M. (2020). Transcriptional and metabolic regulation of grape berry metabolism upon powdery mildew infection.

Authors' Contributions: Ana Margarida Fortes designed and directed the study. Diana Pimentel, Cecília Rego and A.M.F. collected the samples. D.P., Rute Amaro and Flávio Soares performed the nucleic acid extractions, real-time PCR and the biochemical assays under supervision of A.M.F. Metabolomic analysis was performed by D.P. and Alexander Erban under close supervision of Joachim Kopka. Transcriptomic analysis was performed by D.P. under close supervision of Nuria Mauri and Jose Martinez-Zapater. Hormonal profiling was performed by Michael Reichelt and Alex Mithöfer. Comprehensive analysis of processed data was carried out by D.P. under close supervision of A.M.F. D.P. wrote the chapter with valuable input from A.M.F. A.M.F, J.K., A.M. provided funding for the experiments.

2.1. Abstract

Powdery mildew (PM) is one of the most dramatic diseases affecting grapevine (*Vitis vinifera* L.). Powdery mildew (PM) is caused by the ascomycete fungus *Erysiphe necator* Schw. (syn. *Uncinula necator*) which is an obligate biotrophic fungus that causes significant reduction in berries' yield and in wine quality. Even though several studies were conducted to understand the basis beyond resistance to PM, the grapevine defenses are a very complex genetic mechanism and responses against PM remains unclear, particularly in infected fruits. In order to study the response mechanisms of grape berries to powdery mildew infection in the early stages of fruit ripening, naturally infected and control grapes from 'Carignan' variety were collected at green (EL33) and *véraison* (EL35) stages and metabolic, transcriptomic and hormonal changes upon PM infection of susceptible grapes were analyzed. Results revealed an induction of defense-related genes as well as the accumulation of stress-related metabolites, such as resveratrol, catechins and gallic acid. However, this activation was not enough to confer resistance. Transcriptional and metabolic reprogramming was more active at the green stage when the infection levels tend to be higher. Reprograming of fatty acid metabolism was also observed trough the accumulation of long-chain saturated fatty acids and up-regulation of genes involved in fatty acid biosynthesis. The involvement of salicylic acid in response against PM was supported by the accumulation of SA as well as the up-regulation of genes involved in SA signaling, such as *EDS1* and *SAG101*. Moreover, this study was the first to analyze the jasmonates' content in PM infected berries revealing a reprogramming of some jasmonates in response to PM. Overall, this study revealed an activation of defensive mechanisms in susceptible grape berries in response to PM infection.

2.2. Introduction

Grapevine (*Vitis vinifera* L.) is a perennial woody plant highly susceptible to several environmental stress conditions and diseases. Fungal infections are the primary cause of the reduction of grape yield and berry quality. Powdery mildew (PM) is one of the most dramatic diseases affecting grape production worldwide. PM is caused by the ascomycete fungus *Erysiphe necator* Schw. (syn. *Uncinula necator* [Schw.] Burr.), an obligate biotrophic fungus which needs access to plant host nutrients for growth and reproduction, affecting infected clusters by reducing their quality and predisposing them to bunch rot infections with deleterious effect on wine quality (Calonnec et al., 2004; Gadoury et al., 2012, 2007). Eurasia-originated *V. vinifera* species are more susceptible to PM than the native North American grapevines (Qiu

et al., 2015). Moreover, resistance to PM was also found in Chinese accessions of non-*vinifera* species, namely *V. romanetti* (Mahanil et al., 2012; Riaz et al., 2011), *V. pseudoreticulata* (Dai et al., 2016; Wan et al., 2007; Wang et al., 1995; Weng et al., 2014) and, *V. piasezkii* (Pap et al., 2016) and Central Asian accessions of *V. vinifera* (Amrine et al., 2015; Coleman et al., 2009; Hoffmann et al., 2008; Riaz et al., 2013). Since most of the cultivars used for wine and table grape production are *V. vinifera*, PM is currently spread to all vinicultural regions, and the control strategy is entirely dependent on the widespread application of sulfur-based and synthetic fungicides resulting in environmental poisoning and health impacts.

During infection, the *E. necator* conidia form an infection structure, appressorium, that generates pressure on the epidermal layers and rupture the cell wall. Afterward, it penetrates the plant cell, and a feeding structure is formed (haustorium) responsible for the dynamic exchanges between the fungus and the host cells (Armijo et al., 2016; Gadoury et al., 2012). In Portugal, the population of *E. necator* shows low genetic diversity (Oliveira and Cunha, 2015). Plants have several defense mechanisms to prevent pathogen penetration and colonization (Jones and Dangl, 2006). The two primary defense responses are the pathogen-associated molecular patterns (PAMP)-triggered immunity (PTI), mainly related to penetration resistance, and effector-triggered immunity (ETI), to restrict pathogen growth and proliferation. Both responses take place consecutively and are interconnected. PM species adapted to a specific host are supposed to release effectors to repress PTI, resulting in effector-triggered susceptibility. In response, host plants have evolved the ETI as a second layer of resistance whereby these PTI-suppressing effector molecules are detected by *R* genes that, in turn, activate several defense responses, including programmed cell death (Gadoury et al., 2012). Most characterized plant *R* genes encode proteins with leucine-rich repeat domains (LRRs), a central nucleotide-binding site (NB), and a variable N-terminus (Jones and Dangl, 2006). Several *R* genes have been identified in some *Vitaceae* species, as well as several other genes involved in PM resistance such as those coding for pathogenesis-related (PR) proteins, including chitinase and *β*-1,3-glucanase, *PR* genes, stilbene synthases, and genes involved in defense signal perception and transduction, including *Enhanced Disease Susceptibility 1 (ESD1)*, WRKY transcription factors and *RING* finger protein gene family (Qiu et al., 2015).

Plant hormones are also essential keys in biotic stress responses, including salicylic acid (SA), jasmonic acid (JA), and ethylene. SA is mainly related to response against biotrophic and hemibiotrophic pathogens, whereas JA and ethylene are central players in resistance to necrotrophic pathogens (Glazebrook, 2005). The basal levels of SA were shown to be higher in resistant *V. aestivalis* cv. Norton than in susceptible *V. vinifera* cv. Cabernet Sauvignon, which

can be related to resistance (Fung et al., 2008). On the other hand, induction of JA and ethylene signaling has been associated with the elicitation of resistance against powdery mildew in grapevine (Belhadj et al., 2008, 2006).

Most of the studies on responses to fungal infections are focused on grapevine leaves, and little is known about the defense mechanisms in fruits. The actual effect of powdery mildew in grape berry and wine quality is controversial, possibly due to the uneven infection along with the clusters. Changes in sugar content of infected berries and resulting wines were reported to both increase (Calonnec et al., 2004; Ough and Berg, 1979) and decrease (Gadoury et al., 2001), as well as the anthocyanin content (Amati et al., 1996; Calonnec et al., 2004; Ough and Berg, 1979; Piermattei et al., 1999).

During ripening, grape berries develop an ontogenic resistance, i.e. age-related resistance. The period of fruit susceptibility is in general small, and resistance to PM in several *V. vinifera* cultivars increases around 2-4 weeks after bloom (Ficke et al., 2004, 2003; Gadoury et al., 2003), which is characterized by a reduction of penetration rate, near-immunity to new infections or to colonization of established colonies, and changes in latent period and sporophore density (Ficke et al., 2003). Nevertheless, Gadoury et al. (2007) observed that berries could still support inconspicuous colonies after the onset of ontogenic resistance, and this was associated with the presence of epiphytic microorganisms, *Botrytis cinerea*, and insects, that are attracted by volatiles such as ethyl acetate, acetic acid, and ethanol.

Despite the various studies performed so far, the mechanisms behind grapevine resistance or susceptibility are very complex, and PM defense responses remain unclear in infected fruits. Therefore, in this study, high throughput technologies combined with targeted approaches were applied to comprehend how extremely susceptible grape berries (*V. vinifera* cv. Carignan) react to natural PM infection at the early stages of ripening. Additionally, we looked in detail at the hormonal reprogramming and identified new markers of infection. Our results suggest activation of defense mechanisms in response to PM infection, as previously observed in leaves (Fung et al., 2008). Nevertheless, certain responses were observed to be differently modulated in berries and leaves, suggesting organ-specific responses. Furthermore, our results not only supported the involvement of salicylic acid in defense against *E. necator* infection but also suggested a modulation of the jasmonates content, often associated with response against necrotrophs.

2.3. Results

2.3.1. Phenotypic assessment and evaluation of main ripening parameters in powdery mildew infected and control grape berries

In order to study the effect of powdery mildew infection on grape berry physiology, infected berry samples were collected at two ripening stages, green (EL33) and *véraison* (EL35), according to the modified E-L system (Coombe, 1995). EL33 stage is characterized by green and firm berries, with low sugar and high organic acids content, and EL35, which corresponds to the onset of ripening, is characterized by berry softening, color changing due to anthocyanin accumulation and increase in sugar content (Conde et al., 2007). Figure 2.1 shows infected clusters at both EL33 and EL35 stages. Infected and control samples were distinguished by visual inspection and real-time PCR, which revealed that the infection level at EL35 has a tendency to be lower than at EL33 (Supplementary Figure S2.1). *E. necator* affects mainly green tissues, and ontogenic resistance is observed in grape berries (Ficke et al., 2003; Gadoury et al., 2003), which can explain the trend to lower the fungal gene expression at the *véraison* stage (Supplementary Figure S2.1).

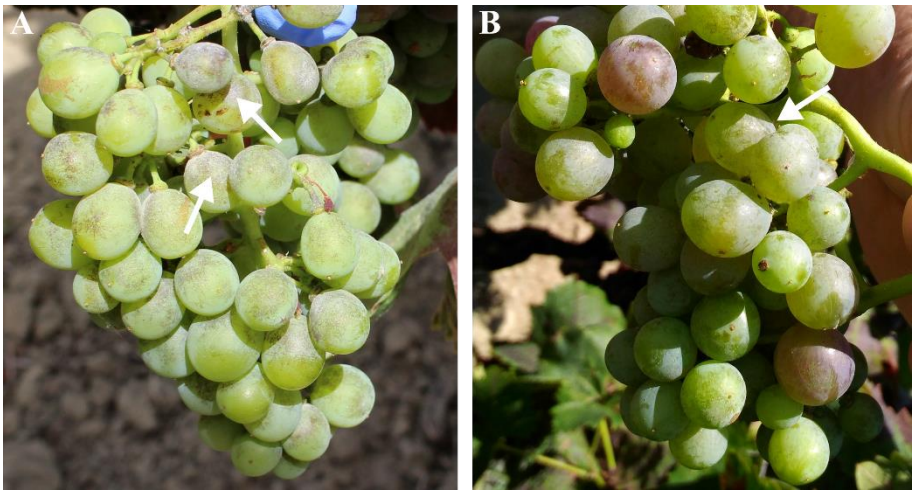

Figure 2.1 - Clusters of *Vitis vinifera* cv. Carignan grapes naturally infected with powdery mildew (*Erysiphe necator*) at (A) EL33 and (B) EL35 developmental stages.

Berry weight and content in main sugars, organic acids, and anthocyanins, were analyzed to evaluate the effect of PM in the main parameters of fruit ripening (Figure 2.2). PM infection led to a higher accumulation of anthocyanins at the green stage; however, no significant alteration was observed for berry weight, sugar, and organic acids between control and infected berries. At *véraison* stage, an increase in anthocyanins, and in glucose and fructose content was observed in both conditions. These results suggest that PM infection caused a minor effect on the main ripening parameters.

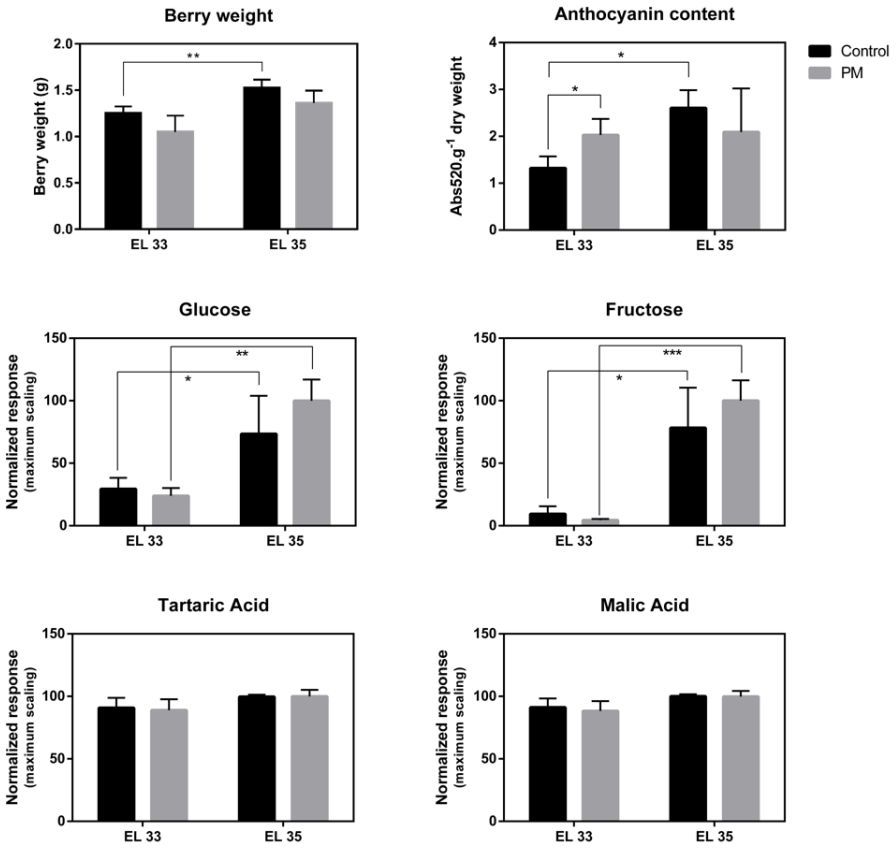

Figure 2.2 - Phenotypic and metabolic characterization of powdery mildew infected (PM) and control grape berries at developmental stages EL33 (green) and EL35 (*véraison*): berry weight, anthocyanin content (absorbance at 520nm g^{-1} of freeze-dried material), and relative quantification of glucose, fructose, tartaric acid, and malic acid (Supplementary Table S2.1). Bars and whiskers represent averages and standard deviation (SD). Significance (*p*-value) of indicated pairwise comparisons was assessed by *Student's t-test*: * *p*-value ≤0.05; ** *p*-value≤0.01; *** *p*-value≤0.001.

2.3.2. Metabolic profiling of control and infected berries revealed a substantial reprogramming of fatty acid metabolism

Metabolic profiling of control and infected berries was performed by gas chromatography coupled to electron impact ionization/time-of-flight mass spectrometry (GC-EI/TOF-MS), which allowed the relative quantification of several classes of compounds, such as fatty acids, sugars, and phenylpropanoids (Supplementary Table S2.1). Moreover, volatile compounds were relatively quantified by solid-phase microextraction (SPME) and gas chromatography coupled to electron impact (GC-EI-MS, Supplementary Table S2.1). One hundred metabolites were identified; twenty-three were volatiles and seventy-seven soluble compounds (Supplementary Table S2.1). Normalized response data was used for Principal Component Analysis (PCA), and the two major PCs explained 37.65% of the variability showing a good separation between control and infected berries and, also, developmental stages (Supplementary Figure S2.2). Thirty-six metabolites showed differential content comparing

either infected and control samples or green and *véraison* stages (Figure 2.3, Supplementary Table S2.1). Additionally, twenty metabolites were identified as positive markers of infection (Figure 2.4), i.e., metabolites that were significantly increased (response ratio ≥ 1.5 and *p*-value ≤ 0.05) or were detected only in infected berries. These metabolites included fatty acids (eicosanoic, docosanoic and tetracosanoic acids), fatty alcohols (eicosan-1-ol, docosan-1-ol, octadecan-1-ol), lipids (α-tocopherol), phenylpropanoids (resveratrol and catechins), phenolic acids (gallic acid), sugar conjugates (4-hydroxyphenyl-beta-glucopyranoside and salicylic acid-glucopyranoside) and an unidentified compound (A255011).

Regarding fatty acids (Figure 2.3), several saturated long-chain fatty acids and fatty alcohols were present in a significantly higher amount in infected berries in comparison to control. Eicosanoic acid (arachidic acid), docosanoic acid (behenic acid), and tetracosanoic acid were accumulated in infected berries at both stages. Modification of fatty acid profile in response to *E. necator* infection has been previously observed in fully developed berries and eicosanoic acid was identified as a specific and quantitative marker of PM presence in grape berries (Petrovic et al., 2017). Hexacosanoic acid, octacosanoic acid, and pentacosanoic acid were also responsive to infection but only at the green stage. Additionally, they were accumulated during *véraison* in control berries (Figure 2.3). The fatty alcohol derived from eicosanoic acid (n-eicosan-1-ol) was only detected in infected berries (Figure 2.4). Long-chain fatty acids are the main components of the plant cuticle, which plays an essential role in plant-pathogen interaction (Ziv et al., 2018). Concerning other acids, fumaric acid, an intermediate metabolite in the citric acid cycle, was present in a lower amount in infected berries at EL33 in comparison to control (Figure 2.3).

Concerning secondary metabolites, gallic acid, epicatechin/catechin, and α-tocopherol were present in higher quantity in infected berries than in control berries at the EL33 stage (Figure 2.3). Gallic acid was shown to have antifungal properties (Lattanzio et al., 2006) and was proposed as a positive marker of *Botrytis cinerea* infection (Agudelo-Romero et al., 2015). Resveratrol was only identified in infected berries (Figure 2.4) confirming other studies (Piermattei et al., 1999; Romero-Pérez et al., 2001). Relative to terpene metabolism, ursolic acid and cycloartenol were present in lower amounts in infected berries at EL35 and both stages, respectively (Figure 2.3). Stigmasterol, a phytosterol involved in plant-pathogen interactions (Griebel and Zeier, 2010), was increased in infected berries at the EL35 stage (Figure 2.3). Salicylic acid-glucopyranoside, a SA sugar conjugated metabolite, was detected only in infected berries at the green stage (Figure 2.4).

Regarding volatile compounds, four metabolites were identified as infection responsive. Limonene, undecane, and phenylacetaldehyde were less accumulated in infected berries at the *véraison* stage than in control berries (Figure 2.3). On the other hand, benzaldehyde was accumulated after infection at the green stage (Figure 2.3).

These results indicate a substantial metabolic reprogramming in berries upon infection with *E. necator*, mostly involving fatty acid metabolism.

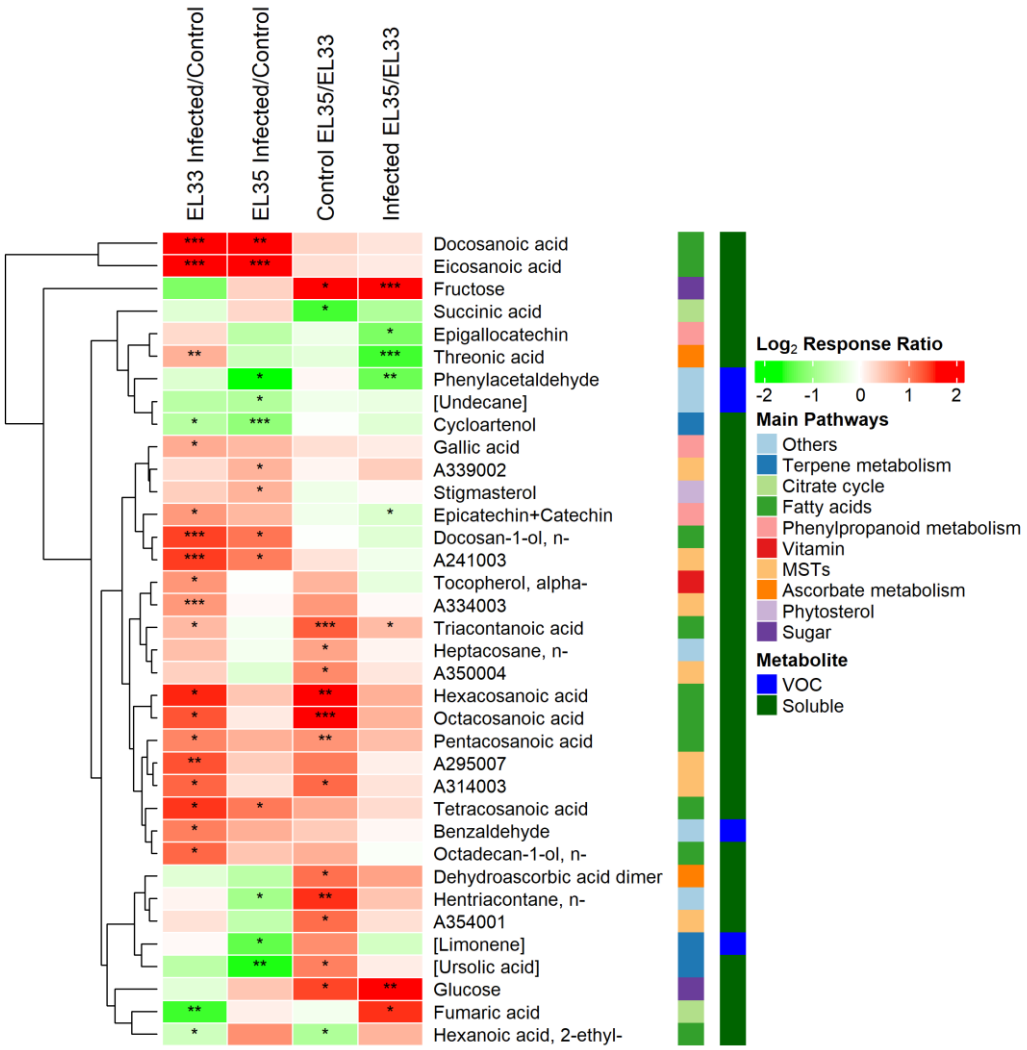

Figure 2.3 - Metabolic analysis of infection- and *véraison*-responsive metabolites from powdery mildew infected and control berries at the green (EL33) and *véraison* (EL35) developmental stages. Metabolites that were significantly increased or decreased in at least one of the pairwise comparisons with response ratio ≥1.5 and *p*-value ≤ 0.05 (cf. statistical tests and analyses of variation in Supplementary Table S2.1) are presented by a heatmap. Metabolites present only in infected berries were not included. Response ratios were $\log_2$-transformed (cf. inserted scale) and hierarchically clustered using Euclidean distance and complete linkage. Asterisks indicate statistical significance (*Student's t-test:* * *p*-value ≤ 0.05; ** *p*-value ≤ 0.01; *** *p*-value ≤ 0.001). MTSs – mass spectral tags.

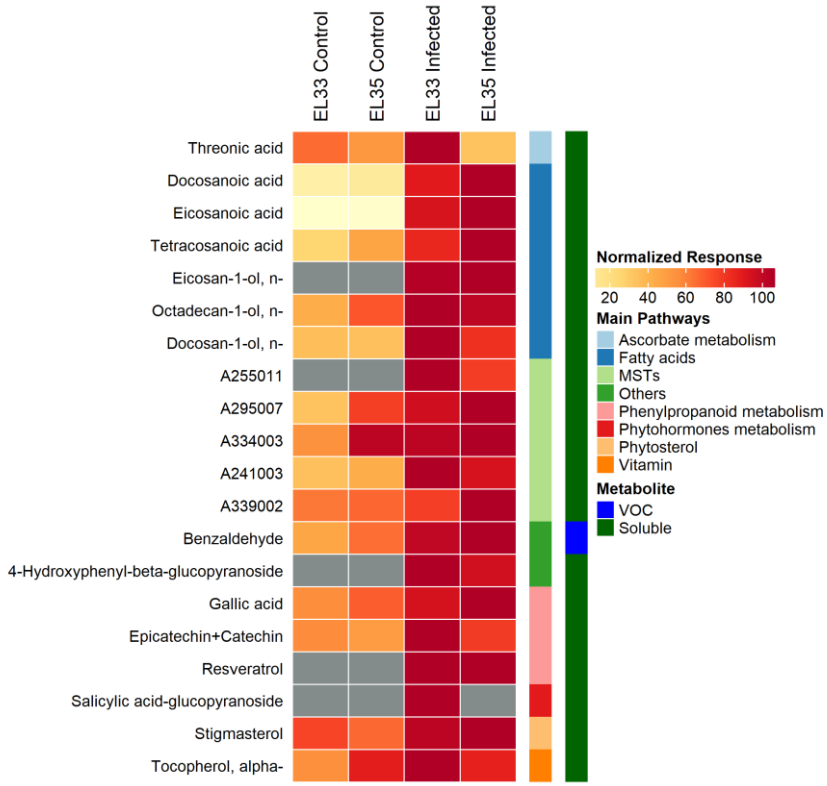

Figure 2.4 - Potential positive markers of powdery mildew infection of berries of *V. vinifera* cv. Carignan at EL33 and EL35 stages. Metabolites selected were either significantly increased after infection at one or both developmental stages (response ratio ≥1.5 and *p*-value ≤ 0.05, *Student's t-test*) or only detected in infected berries. The heatmap represents normalized responses in a two-color scale, low (light orange), high (dark red), grey boxes indicate non-detected metabolites. MTSs – mass spectral tags.

2.3.3. Transcriptional profiling of infected and control grape berry samples

2.3.3.1. Functional enrichment analysis

Functional enrichment analysis of up- and down-regulated transcripts was carried out (Figure 2.5, Supplementary Figure S2.4, Supplementary Table S2.3) based on functional categories defined by Grimplet et al. (2012). Two comparisons were considered: transcriptional changes between control and infected berries (Figure 2.5) and, also, during ripening (EL35 in comparison to EL33, Supplementary Figure S2.4).

Genes that were up-regulated in infected berries at both stages (Figure 2.5) are mainly related to signaling pathways and protein kinases, secondary metabolism (including phenylpropanoids, stilbenoids, and lignin biosynthesis), stress response (such as biotic stress response, plant-pathogen interaction, oxidative stress), hormone signaling (in particular salicylic acid signaling), nitrogen metabolism, phytoalexin biosynthesis, NBS-LRR superfamily, and WRKY transcription factor family. Activation of secondary metabolism and

defense/stress responses was also observed in *V. vinifera* leaves infected with *E. necator* (Fekete et al., 2009; Fung et al., 2008).

Up-regulated functional categories enriched only at the EL33 stage included genes related to transporters, primary metabolism (glutathione metabolism), secondary metabolism (aromatic compound glycosylation, flavonoid, and isoflavonoid biosynthesis), MYB transcription factor family, ABA-mediated signaling and brassinosteroid-mediated signaling (Figure 2.5, Supplementary Table S2.3). Additionally, aromatic amino acid metabolism, lipid metabolism (glycerolipid catabolism), and subtilase-mediated proteolysis were categories enriched only at the EL35 stage (Figure 2.5, Supplementary Table S2.3).

At the green stage, very few categories were enriched as down-regulated (116 genes, Supplementary Table S2.2); they were associated with cell growth, cell wall organization and biogenesis (including cell wall metabolism, modification, and pectin metabolism,), cellular metabolism (cytochrome P450 oxidoreductase), and carbohydrate metabolism (Figure 2.5, Supplementary Table S2.3). Moreover, no functional category was enriched at the *véraison* stage since only 28 genes were down-regulated (Figure 2.5, Supplementary Tables S2.2 and S2.3).

Additionally, real-time PCR analysis was performed (Supplementary Figure S2.6) and validated RNA-seq results (Supplementary Figure S2.7).

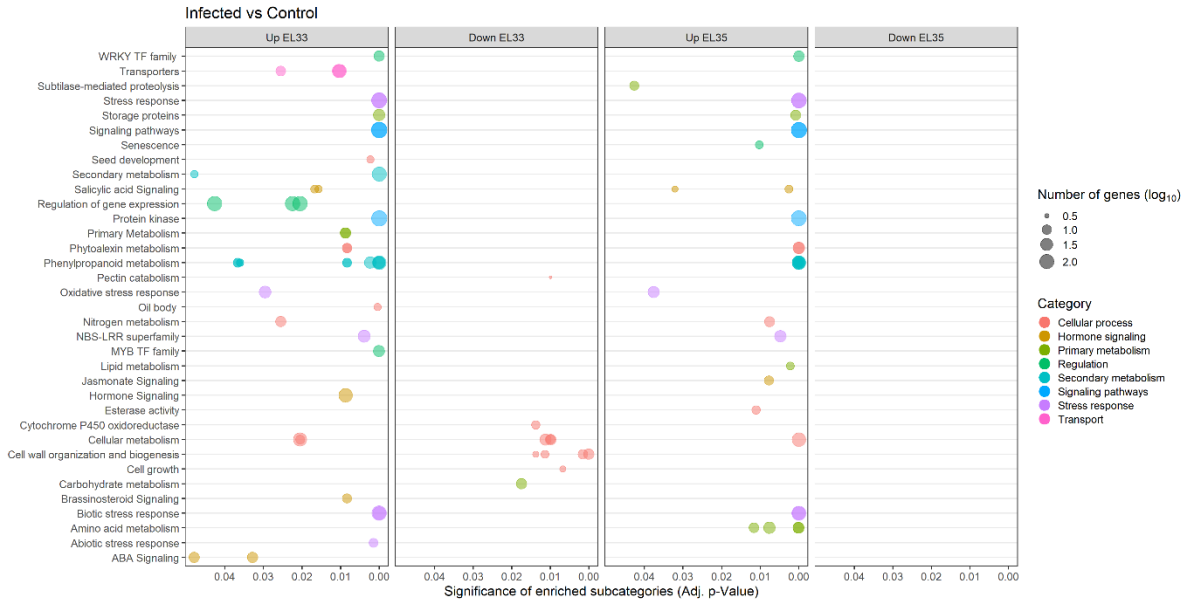

Figure 2.5 - Enriched functional subcategories (adjusted *p*-value ≤ 0.05) in response to PM infection (Infected vs Control). Circles' size represents the number of genes (log10) for each functional subcategory. Complete dataset in Supplementary Table S2.3.

2.3.3.2. Modulation of genes involved in biotic stress responses indicates an early activation of signaling events in non-ripe infected fruits

Genes involved in biotic stress responses were vastly modulated in response to infection (Supplementary Table S2.2). Several genes encoding protein and receptor-like kinases were up-regulated in both stages including several LRR-RKs (leucine-rich repeat receptor-like kinase), RTSKs (receptor serine/threonine kinase), *ARK3* (*Arabidopsis Receptor Kinase 3*), *CLV1* (*Clavata1 receptor kinase*), *CRK10* (*cysteine-rich RLK10*), *LRK10*, *FRK1* (*FLG22-induced receptor-like kinase 1*), *CPK13* (*Calcium-dependent protein kinase 13*) and S-receptor protein kinases (Table 2.1, Supplementary Table S2.2). Several receptor kinases recognize PAMPs or effectors released by the fungus activating plant immunity (Jones and Dangl, 2006). A number of *WAK* genes (*wall-associated receptor kinase*), *BAK1* genes (*Brassinosteroid insensitive 1-associated receptor kinase 1*), one LysM protein (chitin elicitor-binding CEBIP LysM domain-containing), and one *FLS2* gene (*Flagellin-sensitive 2*), which code for PAMPs receptors leading to the activation of defense responses, were activated in response to PM (Table 2.1, Supplementary Table S2.2). A large number of genes belonging to the NBS-LRR superfamily, including TIR-NBS-LRR, EIX receptor, and HcrVf1 protein, and other R proteins (PRF, L6, and MLA10) were over-expressed in response to infection (Table 2.1, Supplementary Table S2.2). Several NBS-LRRs were shown to be involved in PM response in grapevine (Goyal et al., 2019). Moreover, genes coding Avr9/Cf-9 induced proteins were also responsive to infection at both stages, which are supposed to regulate the hypersensitive response during biotic stress (Rowland et al., 2005). Avr9/Cf-9 induced proteins coding genes were also responsive to infection in leaves (Toth et al., 2016). Two MAPK coding genes, MAPKKK5 and MAP4K *alpha*1, were induced in infected berries at the green stage (Table 2.1, Supplementary Table S2.2).

Additionally, several other genes widely described as involved in biotic stress responses were activated such as those coding for PR-10 (pathogenesis-related protein 10), thaumatin, calmodulin and calmodulin-binding proteins, CNGC (cyclic nucleotide-gated ion channel), peroxidases and glutathione S-transferases (GSTs) (Table 2.1, Supplementary Table S2.2).

Myb and WRKY were the most responsive transcription factor gene families to PM infection (Table 2.1, Supplementary Table S2.2). Myb transcription factor family is known to be associated with the regulation of secondary metabolites biosynthesis (Czemmel et al., 2012), and some WRKY transcription factors were shown to be involved in SA signaling pathway enhancing hypersensitive response on host infected cells (Wang et al., 2017). Other transcription factor families with PM-responsive members, particularly at the green stage, were

the *Lateral Organ Boundary (LOB) domain* family and the Zinc finger C3HC4 family (Table 2.1, Supplementary Table S2.2). Several *LOB domain* genes were previously associated with response to powdery mildew (Grimplet et al., 2017).

Cell wall metabolism was also responsive to PM infection, particularly at EL33 stage. Genes involved in pectin catabolism and modification, xyloglucan modification, cell wall catabolism and loosening were down-regulated in response to PM infection (Table 2.1, Supplementary Table S2.2). On the other hand, cellulose synthase and endo-1,4-*β*-glucanase (*KORRIGAN*) were up-regulated in infected berries (Table 2.1, Supplementary Table S2.2). Additionally, three chitinases (class I and IV), one acidic chitinase III, and three *β*-1,3-glucanases coding genes were up-regulated in infected berries, particularly at the green stage (Table 2.1, Supplementary Table S2.2). Chitinases and *β*-1,3-glucanases are secreted by host plants to degrade fungal cell wall into small fragments that will be perceived by plant receptors (such as LysM receptor kinases) to initiate additional defense responses (Brulé et al., 2019).

Additionally, six *MLO* (*Mildew Locus O*) genes (*S*-genes) were up-regulated in infected berries at both stages (Table 2.1, Supplementary Table S2.2). *MLO* genes are associated with susceptibility to powdery mildew in grapevine (Pessina et al., 2016).

Table 2.1 - Selection of infection-responsive genes in powdery mildew (PM) infected and control grape berries (considering an FDR of ≤0.05 and a fold change of ≥ 2 or ≤ –2). Complete dataset in Supplementary Table S2.2.

Gene ID	EL33 (PM vs Control)	EL35 (PM vs Control)	Control (EL35 vs EL33)	PM Infected (EL35 vs EL33)	Functional annotation
			Signaling		
VIT_19s0014g04060	18.6	5.2			ARK3 (Arabidopsis Receptor Kinase 3)
VIT_16s0148g00100	86.0	16.5			Brassinosteroid insensitive 1-associated receptor kinase 1
VIT_03s0038g03220		2.7	-3.2		Chitin elicitor-binding CEBIP LysM domain-containing
VIT_04s0008g00330	45.6	7.5			Clavata1 receptor kinase (CLV1)
VIT_00s2485g00010	32.7	15.3			CRK10 (cysteine-rich RLK10)
VIT_14s0066g00760	11.8	6.7			Disease resistance protein (NBS-LRR class)
VIT_07s0197g00130		2.8			Disease resistance protein (TIR-NBS-LRR class)
VIT_16s0050g01980	5.5	6.6			EIX receptor 2
VIT_01s0010g00380	54.8	9.0			FLS2 (flagellin-sensitive 2)
VIT_09s0002g03010	47.3	11.8		-4.3	FRK1 (FLG22-induced receptor-like kinase 1)
VIT_00s0400g00020	4.0	4.7			HcrVf1 protein
VIT_12s0035g00150	8.9	3.8		-2.1	Leucine Rich Repeat receptor-like kinase
VIT_09s0018g00830	29.4	11.7			Leucine-rich repeat family
VIT_18s0001g13590	50.1				Leucine-rich repeat protein kinase
VIT_18s0072g00980		5.0			Leucine-rich repeat protein kinase
VIT_01s0127g00690	13.6			-8.8	MAP4K alpha1
VIT_18s0001g11240	3.4				MAPKKK5 (Mitogen-activated protein kinase kinase kinase 5)
VIT_15s0024g00400	3.2	6.5			R protein MLA10
VIT_00s0258g00130	60.4	14.5			Receptor kinase homolog LRK10

VIT_16s0148g00370	191.3	127.2			Receptor serine/threonine kinase
VIT_10s0003g02010	57.3				RKF1 (receptor-like kinase in flowers 1)
VIT_19s0014g00810	6.7	5.5	-2.9	-3.6	RKF1 (receptor-like kinase in flowers 1)
VIT_19s0014g04080	16.1	165.6			Serine/threonine-protein kinase receptor ARK3
VIT_12s0028g03520	79.0	39.1			S-receptor kinase
VIT_17s0000g04400	33.8	12.6			Wall-associated kinase 1 (WAK1)
Biotic stress response					
VIT_14s0030g02150	477.5	135.5		-4.6	Calmodulin
VIT_17s0000g03370	9.5	22.3			Calmodulin-binding protein
VIT_06s0004g02670	2.7				Cyclic nucleotide-gated ion channel 15
VIT_08s0040g01770	4.4	2.9			Cyclic nucleotide-gated ion channel 15
VIT_08s0040g00920	130.2	34.1		-7.4	Glutathione S-transferase 25 GSTU7
VIT_12s0035g02100	96.2				Glutathione S-transferase Z1 GSTZ1
VIT_06s0004g03120	136.9	18.9		-4.8	MLO-like protein 3
VIT_05s0077g01530	15.0	5.9			Pathogenesis protein 10 [Vitis vinifera]
VIT_08s0058g00990	17.5	15.9		-4.9	Peroxidase
VIT_14s0068g01920		14.1			Peroxidase
VIT_02s0025g04270		12.6			Thaumatin
VIT_02s0025g04230	7.2	14.3			Thaumatin [Vitis vinifera]
Transcription factors					
VIT_08s0056g01650	19.9			-15.8	Lateral organ boundaries domain protein 20 (LBD20)
VIT_16s0050g00050	130.6				Myb domain protein 18
VIT_19s0085g00050	27.5	14.8			Myb domain protein 58
VIT_00s1624g00010	51.9			-8.9	Myb family
VIT_13s0067g01880		5.6		4.0	Other LOB domain-containing protein ASL5
VIT_13s0067g03130		12.0		4.2	WRKY DNA-binding protein 55
VIT_08s0058g01390	13.4	11.1			WRKY DNA-binding protein 70
VIT_14s0068g01770	66.3				WRKY DNA-binding protein 75
VIT_07s0031g00380	95.0			-11.3	Zinc finger (C3HC4-type ring finger)
VIT_12s0028g02530	17.3	8.4			Zinc finger (C3HC4-type ring finger)
Cell wall and chitinases					
VIT_15s0046g01580	91.6				Acidic chitinase III
VIT_15s0046g01570		2.8	-3.0		Acidic endochitinase (CHIB1)
VIT_08s0007g06060	15.6	19.7			Beta 1-3 glucanase
VIT_01s0137g00430	-3.1			3.2	Cellulase
VIT_00s0531g00060	229.6				Cellulose synthase CSLE1
VIT_02s0025g01920	5.8	15.0	-5.5	-2.1	Cellulose synthase CSLG3
VIT_05s0094g00320	53.5	124.6			Chitinase, class IV [Vitis vinifera]
VIT_00s2526g00010	7.8	4.3			Endo-1,4-beta-glucanase korrigan (KOR)
VIT_13s0067g02930	-9.1			15.4	Expansin [Vitis labrusca x Vitis vinifera] EXPA8
VIT_16s0039g00260	-8.6			5.2	Pectate lyase
VIT_16s0022g00940	-16.6		6.0	94.0	Pectinesterase PME3
VIT_06s0061g00550	-17.2			62.4	Xyloglucan endotransglucosylase/hydrolase 32
Nitrogen metabolism					
VIT_08s0058g00140	31.6	5.8		-5.5	Ammonium transporter 2
VIT_08s0007g03240	4.4			-5.3	Carbonic anhydrase precursor
VIT_05s0020g03280	80.2	43.4			Copper amine oxidase
VIT_07s0005g00530	42.7				NADH glutamate synthase
VIT_01s0127g00070	6.1	4.2			Nitrate transporter2.5
VIT_03s0063g01250	3.2				Nodulin 1A, Senescence-associated
VIT_08s0007g04860	3.3			-4.9	Nodulin family protein
VIT_13s0019g05070	30.4	11.5			Nodulin family protein
Carbohydrate metabolism					
VIT_17s0000g03280		2.0	1.8	4.5	Alcohol dehydrogenase 7

VIT_03s0063g00400		2.0			Alpha-amylase / 1,4-alpha-D-glucan glucanohydrolase
VIT_10s0071g01120	26.3			-42.0	Alpha-galactosidase
VIT_04s0008g01140	42.6	82.6			Beta-fructosidase (BFRUCT1)
VIT_18s0001g02230	185.2			-10.2	Beta-galactosidase / lactase
VIT_00s0895g00010	19.4			-3.2	Glucan 1,3-beta-glucosidase
VIT_16s0013g01950		3.7			Hexose transporter [Vitis vinifera]
VIT_10s0003g03930	2.6	1.8	-1.9	-2.7	Inositol transporter 2
VIT_04s0044g00210	162.4		14.6		Mannitol dehydrogenase
VIT_03s0063g02250	5.1	2.6		-3.6	Polyol transporter 5
VIT_12s0057g00130	64.7			-11.1	Sucrose synthase 2
VIT_05s0020g03140	9.3				Sugar transporter 13
VIT_01s0011g05960	2.1				Trehalose-phosphatase
Lipid metabolism					
VIT_18s0001g04750	48.5			-9.1	Acetylcholinesterase
VIT_04s0079g00790		6.0			Acyl-CoA synthetases (Acyl-activating enzyme 11)
VIT_07s0005g01760		12.7			Glycerol-3-phosphate acyltransferase 3 (AtGPAT3)
VIT_14s0219g00280	3.7			-4.8	Glycerol-3-phosphate dehydrogenase (NAD+)
VIT_12s0028g01180	32.8		5.7		Non-specific lipid-transfer protein
VIT_06s0004g01250	33.9			-9.3	Omega-6 fatty acid desaturase, endoplasmic reticulum (FAD2)
VIT_14s0108g00520	452.4		519.9		Protease inhibitor/seed storage/lipid transfer protein (LTP)
VIT_12s0028g02000	2.4		-2.0	-3.1	Triacylglycerol lipase
Secondary metabolism					
VIT_03s0017g02110	4.3	6.5	-3.4		Anthocyanidin 3-O-glucosyltransferase
VIT_19s0085g00750	12.8			-8.4	Anthocyanidin 3-O-glucosyltransferase
VIT_09s0018g01190	8.2	6.5	-2.2	-2.8	Anthranilate N-benzoyltransferase
VIT_10s0003g00900	3.2			-2.3	Anthranilate N-hydroxycinnamoyl/benzoyltransferase
VIT_16s0098g00850	2.1	1.9	-1.8	-2.0	Caffeic acid O-methyltransferase
VIT_03s0063g00140	10.4	11.6	-3.3	-2.9	Caffeoyl-CoA O-methyltransferase
VIT_13s0067g03820	2.6				Chalcone--flavonone isomerase (Chalcone isomerase)
VIT_09s0070g00240	4.9	5.9	-2.9	-2.4	Cinnamoyl-CoA reductase
VIT_07s0031g01380	53.4	29.2			Ferulate 5-hydroxylase
VIT_07s0031g01370	25.8	3.8		-5.4	Flavonoid 3-monooxygenase
VIT_13s0047g00210	3.7	3.2			Flavonol synthase
VIT_12s0028g01880	150.1	9.9		-9.8	Isoflavone methyltransferase/ Orcinol O-methyltransferase 1 oomt1
VIT_07s0031g03070	417.9			-18.4	Isoflavone reductase
VIT_08s0040g01710	7.8	9.0	-2.9	-2.6	Phenylalanine ammonia-lyase
VIT_11s0016g01640	14.5				Phenylalanine ammonia-lyase
VIT_16s0039g01300		112.7			Phenylalanine ammonia-lyase [Vitis vinifera]
VIT_16s0100g00770	14.3				Stilbene synthase
VIT_16s0100g01010	18.7	10.8			Stilbene synthase
VIT_10s0042g00870		97.9			Stilbene synthase [Vitis vinifera]
VIT_06s0004g08150	3.3	1.8		-2.4	Trans-cinnamate 4-monooxygenase
VIT_18s0041g00840	2.5			-4.3	UDP-glucose: anthocyanidin 5,3-O-glucosyltransferase
VIT_05s0062g00350	56.5				UDP-glucose:flavonoid 7-O-glucosyltransferase
VIT_05s0062g00520	3.9	5.8	-5.9	-4.0	UDP-glucose:flavonoid 7-O-glucosyltransferase
Hormone metabolism					
Salicylic and Jasmonic acid					
VIT_18s0001g11630	2.4		10.1	3.3	Allene oxide synthase
VIT_07s0141g00890	16.7	24.7			CYP94A1
VIT_17s0000g07370	2.7	3.3			EDS1 (Enhanced disease susceptibility 1)
VIT_17s0000g07420		6.4	-2.9		EDS1 (Enhanced disease susceptibility 1)
VIT_06s0004g01500	45.7				Lipoxygenase (LOX2)

VIT_06s0004g01480	6.1			-4.5	Lipoxygenase LOX1
VIT_00s0253g00170	33.9				Methyl jasmonate esterase
VIT_03s0088g00710	79.4	23.6			Pathogenesis-related protein 1 precursor (PRP 1)
VIT_01s0011g05920	4.7				S-adenosyl-L-methionine:salicylic acid carboxyl methyltransferase
VIT_00s0684g00030	2.6			-3.3	SEN1 (dark inducible 1)
Auxin					
VIT_13s0067g00330	3.8				AUX1 auxin influx carrier protein
VIT_08s0040g01220		3.9			Auxin efflux carrier protein 10
VIT_12s0057g00420	6.7	6.0			Auxin-responsive protein AIR12
VIT_07s0005g04380	2.0			-2.4	IAA12
VIT_05s0094g01010	3.4			-3.3	Indole-3-acetate beta-glucosyltransferase
VIT_19s0014g04690	3.6				Indole-3-acetic acid-amido synthetase
VIT_11s0052g00440	29.6			-33.2	PIN1 auxin transport protein
VIT_04s0008g02800	-17.0		12.5	241.7	SAUR_D
VIT_04s0008g06350	59.2				TPR1 (topless-related 1)
Abscisic acid (ABA)					
VIT_02s0087g00910	2.1				9-cis-epoxycarotenoid dioxygenase
VIT_01s0026g02190		2.6			ABA-responsive protein (HVA22c)
VIT_06s0080g00340	129.3			-11.5	ABI5 (ABA insensitive 5)
VIT_07s0005g05400	22.7				Abscisic acid-insensitive protein 3 (ABI3)
VIT_14s0006g03250	8.5				AWPM-19
Ethylene					
VIT_01s0011g03070	4.8				AP2 domain-containing protein RAP2.8
VIT_00s0632g00010		2.8			AP2 domain-containing transcription factor TINY
VIT_05s0077g01860	7.6				ERF (ethylene response factor) sub B-2
VIT_05s0049g00510		3.7			Ethylene response factor ERF1
VIT_13s0019g00540	9.7	7.2		-5.2	Ethylene-responsive protein

2.3.3.3. Genes involved in primary and secondary metabolisms are extensively modulated upon infection

Several genes involved in primary metabolism were responsive to PM infection. Nitrogen metabolism, a functional category enriched as up-regulated at EL33, included genes coding for ammonium transporters, nitrate transporter 2.5, nodulin proteins, a copper amine oxidase gene, NADH glutamate synthase, and a carbonic anhydrase (Table 2.1, Supplementary Table S2.2), suggesting a modulation of host nitrogen transport and metabolism that might be eventually in favor to the fungus. *E. necator* genome lacks several enzymes involved in nitrate metabolism, as well as in sulfate and secondary metabolism, which is suggested to be related to adaptation to obligate biotrophy (Jones et al., 2014).

Although carbohydrate metabolism category was enriched as down-regulated in green infected berries due to an over-representation of genes involved in cell wall metabolism (Supplementary Table S2.3), several genes involved in sugar metabolism were up-regulated and may play an important role in supplying energy and/ or precursors for defensive mechanisms. These included genes related to glycolysis and gluconeogenesis (L-lactate dehydrogenase, aldose 1-epimerase, bisphosphoglycerate mutase, and fructose-bisphosphate

aldolase), monosaccharide metabolism (including mannitol dehydrogenase and *alpha*-galactosidase), starch and sucrose metabolism (*alpha*-amylase, sucrose synthase, and glucan 1,3-*beta*-glucosidase), trehalose metabolism, and polyol and sugar transporters (Table 2.1, Supplementary Table S2.2). Enhanced gene expression of hexose transporter genes in response to PM infection has been previously reported in grape leaves, as well as the activity of the cell wall invertase (Hayes et al., 2010).

Moreover, genes encoding enzymes involved in fatty acid biosynthesis (acyl-CoA synthetase and omega-6 fatty acid desaturase), glycerophospholipids metabolism (triacylglycerol lipases, acetylcholinesterase, glycerol-3-phosphate dehydrogenase, and glycerol-3-phosphate acyltransferase 3) and lipid transport were also responsive to infection (Table 2.1, Supplementary Table S2.2), which is in accordance with the accumulation of fatty acids revealed in the metabolic profiling. Strikingly, previous studies in leaves revealed a down-regulation of the fatty acid biosynthesis pathway and an activation of the fatty acid degradation, suggesting a remobilization of the precursor acetyl-CoA to the isoprenoid biosynthesis (Fung et al., 2008).

Secondary metabolism was also extensively reprogrammed upon infection involving activation of phytoalexin and phenylpropanoid metabolisms (Figure 2.6). The well-known activation of resveratrol synthesis in response to powdery mildew infection (Schnee et al., 2008) was supported by the up-regulation of several genes encoding stilbene synthases (Table 2.1, Figure 2.6, Supplementary Table S2). Reprogramming of the phenylpropanoid pathway was also observed in leaves of susceptible *V. vinifera* cv. Cabernet Sauvignon (Fung et al., 2008) and involved up-regulation of genes coding for PAL enzyme (Table 2.1, Figure 2.6, Supplementary Table S2.2). Nevertheless, its activity and total phenolic content showed no significant differences between infected and control berries (Supplementary Figure S2.8), suggesting that specific isoenzymes are being responsible for the increase of the phenylpropanoids and phenolic acids (Figure 2.3). Flavonoid pathway was also stimulated in response to PM, including genes such as chalcone synthase (CHS), flavonol synthase (FLS), flavonoid 3-monooxygenase (F3'H), Isoflavone reductase, and OOMT1s genes (Table 2.1, Figure 2.6, Supplementary Table S2.2).

Genes encoding enzymes from the hydroxycinnamate and lignin biosynthetic pathways were also up-regulated, including Caffeic acid O-methyltransferase (COMT), Caffeoyl-CoA O-methyltransferase (CCOMT), ferulate 5-hydroxylase (F5H), and laccases (Table 2.1, Figure 2.6, Supplementary Table S2.2). Both hydroxycinnamates and lignins are known to be involved in the reinforcement of the plant cell wall (Faulds and Williamson, 1999; Miedes et al., 2014),

and enhanced expression of genes involved in lignin biosynthesis was also observed in leaves infected with PM (Toth et al., 2016).

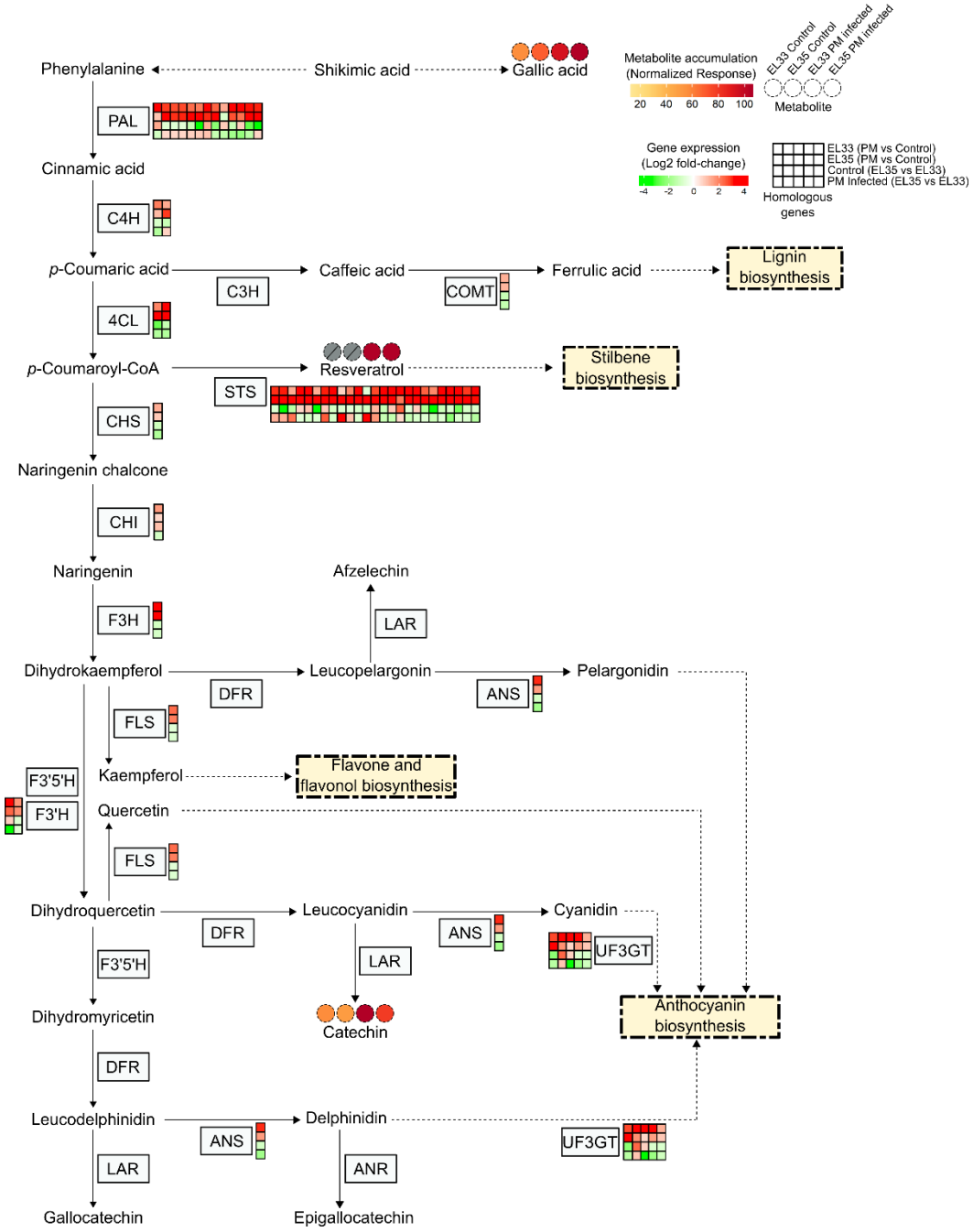

Figure 2.6 - Activation of phenylpropanoid metabolism in response to powdery mildew (PM) infection. Phenylpropanoid and flavonoid pathway representation based on KEGG pathways (www.genome.jp/kegg/pathway.html, last accessed 9 October 2019). Dashed lines represent omitted steps. Heatmap colors represent the gene expression (log_2 fold change) for each comparison (Supplementary Table S2.2). Circles represent metabolites and colors the normalize response in each condition (Supplementary Table S2.1). Crossed grey circles represent non-detected metabolites. EL33, green stage; EL35, *véraison* stage; PAL, phenylalanine ammonia-lyase; C4H, *trans*-cinnamate 4-monooxygenase; C3H, p-coumarate 3-hydroxylase; COMT, caffeic acid 3-*O*-methyltransferase; 4CL, 4-coumaroyl-CoA ligase; CHS, chalcone synthase; STS, stilbene synthase; CHI, chalcone isomerase; F3H, flavonone 3-hydroxylase; DFR, dihydroflavanol 4-reductase; LAR, leucoanthocyanidin reductase; ANS, anthocyanidin synthase; ANR, anthocyanidin reductase; FLS, flavonol synthase; F3'5'H, flavonoid 3',5'-hydroxylase; F3'H, flavonoid 3'-monooxygenase; UF3GT, UDP-glucose:anthocyanidin 3-*O*-d-glucosyltransferase.

2.3.3.4. Modulation of genes involved in hormonal metabolism highlights the central role of salicylic acid signaling in response to powdery mildew

Around eighty genes involved in hormonal metabolism were shown to be infection-responsive in green and/or *véraison* stages. Regarding the salicylic acid (SA) pathway, an over-expression of the *EDS1* and *PR-1* genes was observed upon infection at both stages (Table 2.1, Supplementary Table S2.2). Up-regulation of *EDS1* in response to *E. necator* infection was also previously observed in leaves (Fung et al., 2008; Gao et al., 2010; Toth et al., 2016). *SAG101* genes, which code for an interaction partner of EDS1 in response to pathogens (Gantner et al., 2019; Wagner et al., 2013; Zhu et al., 2011), were also up-regulated in infected berries (Table 2.1, Supplementary Table S2.2). Moreover, a *SAMT* gene, which codes a salicylate *O*-methyltransferase that catalyzes the conversion of SA to methyl salicylate (MeSA), was up-regulated in response to PM infection, mainly at green stage (Table 2.1, Supplementary Table S2.2). Another gene also coding for this enzyme was reported to be up-regulated in response to both PM infection and MeSA treatment in leaves (Toth et al., 2016), indicating that might exist an organ specificity of the activated genes.

Concerning jasmonic acid (JA) metabolism, genes encoding methyl jasmonate esterase, involved in methyl-JA degradation, lipoxygenases (LOX), and an allene oxide synthase (AOS) were up-regulated at green infected berries (Table 2.1, Supplementary Table S2.2). Up-regulation of the *AOS* and *LOX2* genes was observed in 'Cabernet Sauvignon' leaves both infected with PM and treated with MeSA (Toth et al., 2016). Additionally, one gene belonging to the cytochrome P450 (CYP94) family was up-regulated in response to PM infection (Table 2.1, Supplementary Table S2.2). Members of the CYP94 family were shown to be involved in the oxidation of the bioactive form of JA, jasmonoyl-L-isoleucine (JA-Ile; Heitz et al., 2012; Koo et al., 2014, 2011).

Regarding auxins, *TPR1* (*topless-related 1*) gene, which is involved in gene repression, was up-regulated in green infected berries, as well as gene coding for auxin transporters, namely *PIN1* and *AUX1*, auxin-responsive proteins and genes involved in auxin metabolism, including, Indole-3-acetic acid-amido synthetase and IAA-*β*-glucosyltransferase (Table 2.1, Supplementary Table S2.2). IAA-amido synthetases are supposed to be involved in auxin homeostasis through amino acid conjugation (Wang and Fu, 2011). Two SAUR genes were down-regulated in response to PM at the green stage (Table 2.1, Supplementary Table S2.2). SA-mediated plant immunity was found to up-regulate certain IAA-amido synthase genes and down-regulate certain *SAUR* genes, as well as AUX1, PIN7, auxin receptors TIR1 and AFB1, and genes from the Aux/IAA family (Wang et al., 2007).

Relative to ABA signaling, genes encoding *ABA insensitive* (*ABI*), AWPM-19 like proteins, ABA-responsive protein (HVA22c), and Protein phosphatase AHG1 were up-regulated at infected berries mostly at EL33 stage (Table 2.1, Supplementary Table S2.2). The expression level of *9-cis-epoxycarotenoid dioxygenase* (*NCED*) gene, involved in ABA biosynthesis, was also increased in response to PM at the green stage.

Concerning ethylene, several members of the AP2 transcription factor family, including ethylene-responsive factors (ERF) genes, were also activated in infected berries (Table 2.1, Supplementary Table S2.2).

2.3.3.5. Fungal metabolic program during infection putatively involves secretion of effectors and Carbohydrate-Active enZymes (CAZymes)

RNA-seq raw data was also aligned with the *E. necator* C-strain genome (Jones et al., 2014) with 5089 (78.5%) of the predicted transcripts detected across all infected berries: 4945 at EL33 and 4040 at EL35 (Supplementary Table S2.4). Several transcripts were detected only in one stage; however, when detected at both developmental stages, no differential expression was observed (Supplementary Table S2.4).

Effectors are secreted by plant pathogens to guarantee a successful infection through manipulation of the host metabolism or defense mechanisms (Ma and Guttman, 2008). Several putative effector genes were expressed at both developmental stages, including those coding for glucose-repressible alcohol dehydrogenase transcriptional effectors, ribonuclease-like proteins, and CSEPs (Candidates for Secreted Effector Proteins); nevertheless, the majority of the EKA (Effectors homologous to *Avrk1* and *Avra10*)-like protein transcripts were detected at EL33 (Table 2.2; Supplementary Table S2.4). These transcripts coding putative effector proteins were also detected at early time points of leaves infection (Jones et al., 2014).

Moreover, several genes coding fungal Carbohydrate-Active enZymes (CAZymes), which are involved in biosynthesis, modification or degradation of carbohydrate bonds (Cantarel et al., 2009), were detected in infected berries at both green and *véraison* stages, including genes coding for carbohydrate esterase, glycosyltransferases, glycoside hydrolases (such as glucanase and glucan-β-glucosidase), and carbohydrate-binding modules (Table 2.2; Supplementary Table S2.4). Fungal CAZymes have a significant role in the modification of the plant cell wall polysaccharides during host-pathogen interaction (Blanco-Ulate et al., 2014; Cantarel et al., 2009). Several CAZyme genes were also expressed in infected leaves (Jones et al., 2014).

Moreover, genes involved in chitin biosynthesis, carbohydrate uptake and metabolism (sucrose/sugar transporters, raffinose synthase and maltose permease), fatty acid metabolism (including esterases, lipases and phospholipases), nitrogen uptake (ammonium transporter and nitrate reductase), cutin degradation (cutinase) were also expressed at both developmental stages (Table 2.2; Supplementary Table S2.4). Genes related to polyamine metabolism, such as argininosuccinate synthetase, ornithine carbamoyltransferase, argininosuccinate lyase, and ornithine decarboxylase, were also detected. Polyamines are involved in a wide variety of processes, including spore germination, appressorium formation and conidiation (reviewed by Rocha and Wilson, 2019).

Genes related to DNA modification, including helicases, and histone acetyltransferases, deacetylases, and demethylases, were also expressed during infection (Table 2.2; Supplementary Table S2.4). Chromatin-based control mechanisms have been shown to regulate the effector gene expression of plant-associated fungi (Soyer et al., 2015).

Table 2.2 - Selection of *Erysiphe necator* genes in powdery mildew (PM) infected and control grape berries. Complete dataset in Supplementary Table S2.4.

Transcript.ID	Functional Annotation	Present in EL33	Present in EL35
	Effectors		
EV44_t0562	celp0028 effector like protein	+	+
EV44_t0361	csep0049 effector protein	+	+
EV44_t0037	csep0242 effector protein	+	
EV44_t0277	eka-like protein	+	
EV44_t2445	eka-like protein	+	
EV44_t5321	glucose-repressible alcohol dehydrogenase transcriptional effector	+	+
EV44_t2234	rna binding effector protein scp160	+	+
EV44_t4585	secreted effector protein	+	+
EV44_t5361	secreted effector protein	+	
EV44_t4200	virulence effector		+
	CAZymes		
EV44_t0515	carbohydrate esterase family 5 protein	+	+
EV44_t2590	carbohydrate-binding module family 48 protein	+	+
EV44_t1121	dolichyl glycosyltransferase	+	+
EV44_t0185	glucan-*beta*-glucosidase	+	+
EV44_t0299	glycoside hydrolase	+	+
EV44_t0133	glycoside hydrolase deacetylase	+	+
EV44_t0070	glycosyltransferase family protein	+	
	Cell wall		
EV44_t3003	cell wall glucanase	+	+
EV44_t0156	endo-*beta*-glucanase	+	+
EV44_t4885	glucan synthesis regulatory protein	+	+
EV44_t0089	gpi-anchored cell wall *beta*-endoglucanase	+	+
EV44_t2782	chitin deacetylase	+	+
EV44_t4424	chitin synthase	+	+
	Carbohydrate metabolism		
EV44_t2476	maltose permease	+	+

EV44_t2456	raffinose synthase sip1	+	+
EV44_t1203	high-affinity glucose transporter	+	+
EV44_t6497	myo-inositol transporter	+	
EV44_t0175	sucrose transporter	+	+
EV44_t6132	sugar transporter	+	+
EV44_t2035	udp-galactose transporter like protein	+	+
	Fatty acid metabolism		
EV44_t0138	extracellular lipase	+	+
EV44_t4768	fatty acid desaturase	+	+
EV44_t5564	fatty acid elongase	+	+
EV44_t2281	fatty acid hydroxylase	+	+
EV44_t1141	fatty acid oxygenase	+	+
EV44_t2517	fatty acid synthase subunit alpha	+	+
EV44_t1033	inositol phosphosphingolipids phospholipase c protein	+	+
EV44_t5122	Lipase	+	+
EV44_t5957	omega-3 fatty acid desaturase	+	+
EV44_t2799	phospholipase d1	+	+
EV44_t0377	triacylglycerol lipase	+	+
EV44_t0030	fatty acid transporter	+	+
	Nitrogen metabolism		
EV44_t5850	ammonium transporter	+	+
EV44_t1219	nitrate reductase	+	+
EV44_t2316	nitrilase family protein	+	+
EV44_t5397	nitrogen response regulator	+	+
	Cutin		
EV44_t0350	Cutinase	+	+
	Polyamine metabolism		
EV44_t2279	argininosuccinate lyase	+	+
EV44_t0887	argininosuccinate synthetase	+	+
EV44_t1419	ornithine carbamoyltransferase	+	+
EV44_t2871	ornithine decarboxylase	+	
EV44_t5809	spermine spermidine synthase family protein	+	+
	DNA modification		
EV44_t1446	histone acetyltransferase esa1	+	+
EV44_t4537	histone chaperone	+	+
EV44_t1305	histone deacetylase	+	+
EV44_t1301	histone lysine methyltransferase set7 protein	+	
EV44_t5676	histone promoter control 2 protein	+	+
EV44_t1760	histone-arginine methyltransferase carm1	+	+
EV44_t5518	histone-fold containing protein	+	+

2.3.4. Phytohormonal analysis indicates the involvement of salicylic acid and jasmonates in response to powdery mildew

Phytohormonal analysis revealed that PM infection caused an accumulation of SA and SA-*β*-D-glucoside at both stages, though more pronounced at EL33. This indicates the involvement of SA metabolism and signaling in grape berry response to PM (Figure 2.7). Moreover, GC-TOF-MS analysis identified salicylic acid-glucopyranoside, a SA sugar conjugated metabolite, as a marker of infection at the green stage (Figure 2.4). SA was associated with defense response to biotrophic fungus by several studies (reviewed by

Glazebrook, 2005), including analyses in grapevine leaves infected with powdery mildew (Fung et al., 2008).

Relative to jasmonates, content in JA and its precursor *cis*-OPDA was not affected by infection (Figure 2.7). However, 12-*O*-glucoside-JA was accumulated in infected berries at both stages with higher levels at the green stage (Figure 2.7), suggesting activation of JA glycosylation in response to PM infection. JA-Ile and OH-JA-Ile showed a tendency to increase in response to PM; however, no differences were observed for COOH-JA-Ile (Figure 2.7). JA-Ile is considered to be a bioactive jasmonate (Staswick and Tiryaki, 2004).

Overall, results not only reinforce the involvement of SA as regulator of defense against *E. necator* but also suggest a reprogramming of the jasmonates' pathway. Additionally, results showed no significant alteration in the content of IAA and ABA in response to PM (Figure 2.7).

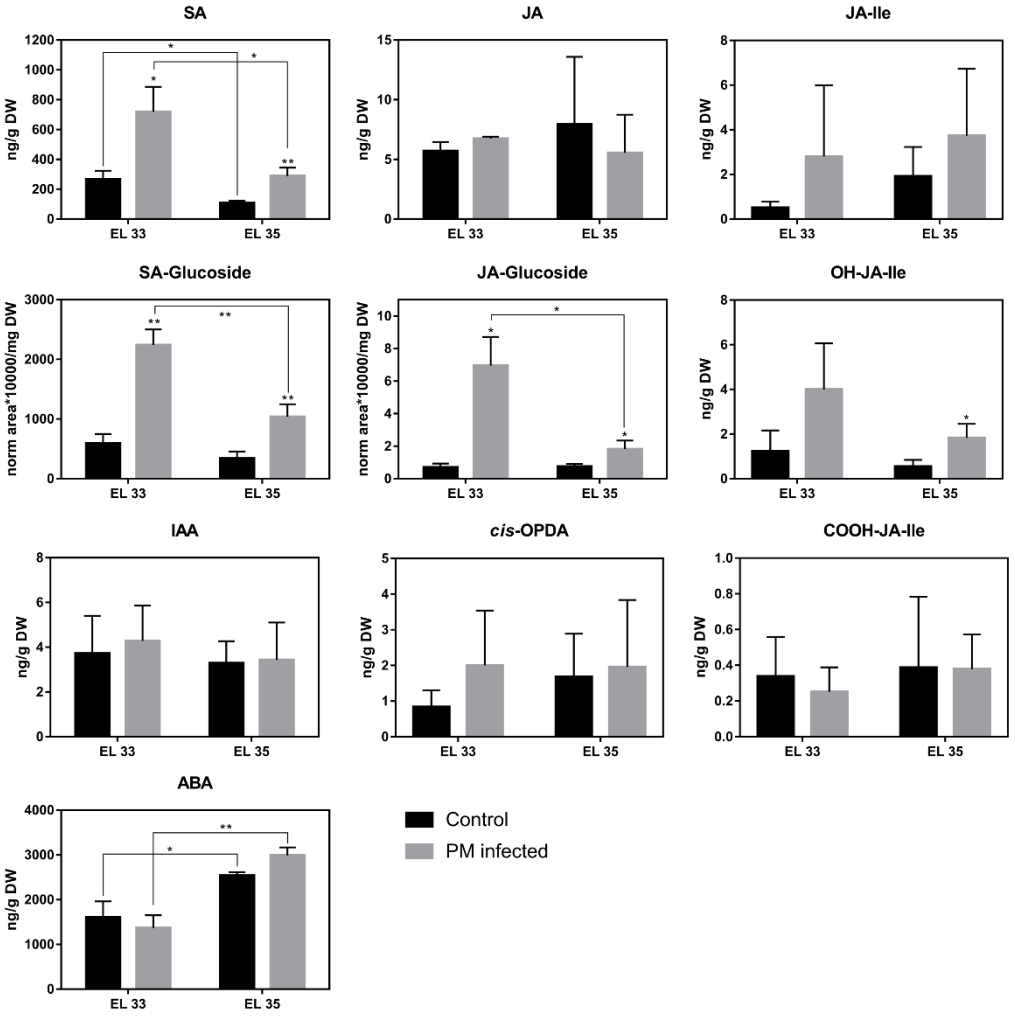

Figure 2.7 - Hormonal profiling of powdery mildew (PM) infected and control grape berries at green (EL33) and *véraison* (EL35) stages. Bars and whiskers represent averages and standard deviation (SD). Asterisks indicate statistical significance on pairwise comparisons (*Student's t-test:* * p-value ≤ 0.05; ** p-value ≤ 0.01). SA, salicylic acid; SA-Glucoside, salicylic acid-*β*-D-glucoside; *cis*-OPDA, 12-oxo-phytodienoic acid; JA, jasmonic acid; JA-Glucoside, 12-*O*-glucoside jasmonic acid; JA-Ile, conjugate jasmonoyl isoleucine; OH-JA-Ile, hydroxyjasmonoyl-isoleucine; COOH-JA-Ile, dicarboxyjasmonoyl-isoleucine; ABA, abscisic acid; IAA, indole acetic acid.

2.3.5. Global changes in the transcriptome and metabolome of ripening berries induced by the fungus

Overall, the main ripening parameters were not affected by PM infection (Figure 2.2), indicating that the onset of ripening was not severely disturbed due to the infection; however, only 31.2% of the genes exhibited the same trend in expression when comparing control (EL35 vs. EL33) and infected berries (EL35 vs. EL33).

At the green stage, anthocyanins accumulated in response to infection; however, no significant difference was observed at the *véraison* stage between control and infected berries, suggesting an anticipation of anthocyanin accumulation typical of the *véraison* stage. Genes related to phenylpropanoid pathway, in particular, the anthocyanin biosynthesis, were shown to be down-regulated at the *véraison* stage in infected berries (Supplementary Figure 2.5). Nevertheless, the expression of some genes was increased at *véraison* during regular ripening (Table 2.3, Supplementary Table S2.2). Therefore, the phenylpropanoid pathway seems to be activated earlier in infected berries than in control. Moreover, several Myb transcription factors, which are known to regulate the phenylpropanoid pathway, were shown to be down-regulated in infected *véraison* berries (Table 2.3, Supplementary Table S2.2). Ursolic acid, a triterpenoid acid, was accumulated at the *véraison* stage under regular ripening; however, infected berries maintained the same level at both stages. Triterpenoid acids usually found in epicuticular fruit waxes, which can function as a barrier against pathogens (Szakiel et al., 2012). Triterpenoid content in grapevine cuticular wax was shown to change during ripening; when total triterpenoids content gradually decreased and a slight increase in the level of neutral triterpenoids was observed (Pensec et al., 2014).

Regarding other transcription factor families, some genes coding for zinc finger (C3HC4-type ring finger), as well as the WRKY51 coding gene, were shown to be repressed during ripening; however, this repression was not observed in infected berries (Table 2.3, Supplementary Table S2.2). On the other hand, the overexpression of two genes coding zinc finger B-box at *véraison* under control condition was affected by PM infection (Table 2.3, Supplementary Table S2.2). Therefore, certain transcription factors that might be involved in the onset of ripening were differently modulated in infected berries.

Phytohormones are key regulators of berry development and ripening (Fortes et al., 2015). Functional enrichment analysis showed that ethylene- and auxin-mediated signaling were functional categories enriched in PM infected berries (Supplementary Figure S2.5). Ethylene and abscisic acid (ABA) are suggested as ripening inducers, whereas auxin inhibits ripening (Fortes et al., 2015). Ethylene-related genes up-regulated during ripening in infected

berries and not differentially expressed in normal ripening conditions included Erg-1, DREB sub A-1 of ERF/AP2 transcription factor (CBF4), Ethylene-responsive transcription factor related to APETALA2 4 coding genes (Table 2.3, Supplementary Table S2.2). *Véraison*-responsive genes in infected berries and not under control conditions related with auxins included SAUR genes and an *alpha*-expansin 6 precursor coding gene (Table 2.3, Supplementary Table S2.2). During regular ripening, Indole-3-acetate beta-glucosyltransferase coding gene was up-regulated and expansin precursors coding genes were down-regulated; however, these differential expressions were affected by PM infection. *EDS1* genes were shown to be repressed during ripening in 'Carignan' berries; however, *E. necator* infection activated the expression of *EDS1* at the *véraison* stage, maintaining the transcript levels during development (Table 2.3, Supplementary Table S2.2). Regarding jasmonate metabolism, methyl jasmonate esterase and lipoxygenase coding genes showed a different trend in expression during ripening when berries were infected (Table 2.3, Supplementary Table S2.2).

PM infection affected during ripening the expression of several genes related to primary metabolism, such as genes associated with amino acid metabolism (tropinone reductase and polyphenol oxidase), photosynthesis, polyamines (ornithine decarboxylase) and carbohydrate metabolisms (Supplementary Figure S2.5, Supplementary Table S2.3). Although no significant differences were observed in the relative quantification of fructose and glucose between control and infected berries, *véraison*-responsive up-regulated genes in infected berries were enriched in genes related to carbohydrate metabolism, in particular, sucrose biosynthesis (Supplementary Figure S2.5, Supplementary Table S2.3), suggesting that the infection might interfere with the typical mechanisms of sugar metabolism during berry development.

Regarding cell wall metabolism, the expression of genes coding pectinesterase, cellulase, polygalacturonase, and xyloglucan endotransglucosylase/ hydrolase were differently modulated when comparing control berries (EL35/EL33) and infected berries (EL35/EL33), suggesting that the reprogramming of cell wall metabolism that occurs during ripening is affected by the presence of the fungus in the berry (Table 2.3, Supplementary Table S2.2).

Related to fatty acid metabolism, hexacosanoic, octacosanoic, and pentacosanoic acids were *véraison*-responsive metabolites (Figure 2.3); however, their accumulation was anticipated in infected berries at the green stage. *E. necator* infection was previously reported to alter the fatty acid profile of fully developed berries (Petrovic et al., 2017). Some genes involved in fatty acid biosynthesis and glycerophospholipid metabolism were down-regulated during berry development in infected berries (Table 2.3, Supplementary Table S2.2).

Several stress-related genes were *véraison*-responsive; nevertheless, some were only differentially expressed under control conditions, including genes associated with abiotic stress (such as dehydrin) and plant-pathogen interaction (R protein L6 and FRK1), suggesting repression of defense mechanisms that are usually activated during ripening (Table 2.3, Supplementary Table S2.2).

Table 2.3 - Selection of véraison-responsive genes in powdery mildew (PM) infected and control grape berries. Asterisks identify differentially expressed genes (considering an FDR of ≤0.05 and a fold change of ≥ 2 or ≤ –2). Complete dataset in Supplementary Table S2.2.

Gene ID	EL33 (PM vs Control)	EL35 (PM vs Control)	Control (EL35 vs EL33)	PM Infected (EL35 vs EL33)	Functional annotation
			Transcription factors		
VIT_11s0016g01320	1.8	-1.0	-1.4	-2.6*	Myb domain protein 13
VIT_06s0061g00470	26.0*	3.7	-1.2	-8.5*	Myb domain protein 36
VIT_13s0067g01360	4.1	-1.5	-1.2	-7.8*	Myb domain protein 77
VIT_00s1624g00010	51.9*	6.5	1.0	-8.9*	Myb family
VIT_07s0031g01710	5.8*	19.4*	-4.1*	-1.2	WRKY DNA-binding protein 51
VIT_00s0347g00030	1.1	-1.2	2.0*	1.5	Zinc finger (B-box type)
VIT_18s0001g03270	-2.8	20.3	-59.5*	-1.1	Zinc finger (C3HC4-type ring finger)
VIT_09s0002g06110	1.3	4.2*	-4.0*	-1.2	Zinc finger (Ran-binding)
			Hormones		
Ethylene					
VIT_18s0001g06030	-1.4	5.5	1.3	10.2*	Erg-1
VIT_16s0100g00380	-2.8	13.4	1.1	45.2*	DREB sub A-1 of ERF/AP2 transcription factor (CBF4)
VIT_02s0025g01360	-1.4	1.4	1.0	2.1*	Ethylene-responsive transcription factor related to APETALA2 4
VIT_18s0001g13320	169.3*	2.1	34.5*	-2.3	Dehydration-responsive element-binding protein 2D
Auxins					
VIT_08s0007g00440	-5.9*	-1.3	1.4	6.6*	Alpha-expansin 6 precursor
VIT_08s0007g05210	-2.0	-1.0	1.3	2.5*	Amino acid permease
VIT_18s0001g00300	-5.8	-1.2	1.4	7.3*	Auxin responsive SAUR protein
VIT_11s0016g00500	-4.7	2.1	-1.8	5.6*	Auxin-responsive SAUR37
VIT_09s0002g08510	-6.8*	-1.6	-5.8*	-1.4	Expansin beta 2 precursor
VIT_06s0004g07230	4.6	-1.6	6.3*	-1.1	Indole-3-acetate beta-glucosyltransferase
Salicylic acid and jasmonates					
VIT_17s0000g07400	1.8	5.2*	-2.8*	1.0	Disease resistance protein (EDS1)
VIT_17s0000g07420	1.9	6.4*	-2.9*	1.1	EDS1 (Enhanced disease susceptibility 1)
VIT_06s0004g01450	1.4	-7.4*	5.4*	-1.8	Lipoxygenase (LOX2)
VIT_00s0253g00080	3.6*	-1.2	2.1*	-1.9	Methyl jasmonate esterase
VIT_13s0064g01480	-2.1	1.4	-4.2*	-1.4	Lipoxygenase LOX1
VIT_00s0253g00090	-1.1	4.2	-6.5*	-1.4	Methyl jasmonate esterase
			Primary Metabolism		
Amino acid metabolism					

VIT_02s0025g03610	9.0*	-1.4	5.3*	-2.4	Phosphoribosylanthranilate transferase
VIT_03s0038g04730	2.9	-2.3	3.8*	-1.7	Polyphenol oxidase
VIT_06s0004g05310	4.5	-1.2	6.9*	1.3	Tropinone reductase
VIT_19s0014g02190	4.1	-1.1	6.0*	1.4	Tyrosine aminotransferase
Photosynthesis					
VIT_17s0000g06350	1.4	-1.2	-1.2	-2.1*	LHCA4 (Photosystem I light harvesting complex gene 4)
VIT_18s0001g08620	1.6	-1.5	-1.3	-3.1*	Photosystem I P700 chlorophyll a apoprotein A2
VIT_19s0015g01760	1.4	-1.3	-1.1	-2.1*	Photosystem I reaction center subunit V (PSAG)
VIT_06s0080g00920	1.6	-1.4	-1.3	-3.1*	Photosystem I subunit O (PSAO)
VIT_13s0019g00320	1.9	-3.7	-1.0	-7.2*	Photosystem II protein P PsbP
VIT_11s0037g00290	-1.1	-7.1	-1.4	-9.3*	Plastocyanin domain-containing protein
Carbohydrate and cell wall					
VIT_19s0138g00110	-1.7	1.2	1.4	2.9*	1,3-beta-glucan synthase
VIT_04s0008g00520	-1.4	1.2	1.3	2.1*	6-phosphofructokinase
VIT_07s0005g04130	-2.2*	1.0	1.2	2.8*	Acid phosphatase class B
VIT_02s0025g00430	-1.1	-1.9	3.4*	1.8	Cellulase
VIT_03s0017g02240	-1.2	2.4	-4.2*	-1.5	Glucan endo-1,3-beta-glucosidase precursor
VIT_13s0158g00130	5.7	1.6	4.2*	1.1	Lactoylglutathione lyase
VIT_04s0044g00210	162.4*	2.1	14.6*	-5.2	Mannitol dehydrogenase
VIT_15s0048g00500	28.6*	1.2	7.0*	-3.2	Pectinesterase family
VIT_09s0054g01080	10.4	1.5	8.7*	1.1	Polygalacturonase QRT3
VIT_13s0019g00840	-1.7	1.0	1.4	2.3*	UDP-glucuronate decarboxylase.
VIT_11s0052g01230	97.9*	-2.0	11.8	-16.7*	Xyloglucan endotransglucosylase/hydrolase 23
VIT_07s0185g00050	15.3	-2.9	40.2*	-1.1	Xyloglucan:xyloglucosyl transferase
Polyamines					
VIT_18s0001g00740	7.7	-4.0	11.4*	-2.7	Ornithine decarboxylase
Fatty acid metabolism					
VIT_17s0000g01090	3.0	1.2	-1.3	-3.1*	Acyl-(acyl carrier protein) thioesterase
VIT_07s0005g05610	-1.9	-1.0	1.1	2.0*	3-ketoacyl-CoA thiolase PED1
VIT_14s0108g01660	2.1	-1.0	-2.0	-4.2*	Biotin carboxyl carrier protein of acetyl-CoA carboxylase
VIT_14s0219g00280	3.7*	1.5	-2.0	-4.8*	Glycerol-3-phosphate dehydrogenase (NAD+)
VIT_13s0019g01990	2.3	1.4	-1.6	-2.7*	Phospholipid/glycerol acyltransferase
VIT_04s0008g05450	1.1	1.8	-2.9*	-1.8	Phospholipase D alpha 1 precursor (PLD 1) (Choline phosphatase 1)
VIT_18s0001g04750	48.5*	-1.2	4.5	-9.1*	Acetylcholinesterase
Secondary metabolism					
VIT_05s0136g00260	1.2	-1.3	2.0*	1.2	Chalcone synthase
VIT_14s0083g00320	2.7*	1.4	2.1*	1.1	Cinnamoyl-CoA reductase
VIT_01s0137g00520	2.4	-1.1	3.2*	1.2	CYP71B35
VIT_07s0031g03070	417.9*	7.1	3.4	-18.4*	Isoflavone reductase
VIT_02s0025g02960	10.5*	1.7	7.5*	1.2	Naringenin,2-oxoglutarate 3-dioxygenase
Stress response					
VIT_17s0000g08050	4.2	-3.9	8.5*	-1.9	Cyclic nucleotide-gated ion channel 16
VIT_03s0038g04390	23.3*	2.7	7.5*	-1.2	Dehydrin 1
VIT_12s0059g01090	1.7	1.1	2.2*	1.5	Early-responsive to dehydration
VIT_09s0002g03020	41.9*	4.8*	3.9*	-2.2	FRK1 (FLG22-induced receptor-like kinase 1)

VIT_08s0007g04240	31.8*	2.9	7.5*	-1.5	Late embryogenesis abundant protein
VIT_12s0034g01350	2.0	1.3	2.1*	1.4	R protein L6
VIT_06s0080g00260	21.0*	2.7	9.4*	1.2	Seed maturation protein PM38

2.4. Discussion

Powdery mildew is one of the most widespread diseases of *Vitis vinifera* plants. Powdery mildew is caused by the obligate biotrophic fungus *E. necator*, which depends on the host cells to complete its life cycle. Most of the studies realized so far were focused on leaves and revealed that susceptible grapevines activate genes associated with defense (Fekete et al., 2009; Fung et al., 2008; Toth et al., 2016). Nevertheless, the understanding of how fruits respond to these biotrophic fungal pathogens will be essential for further grapevine improvement, since responses can be organ-specific. In fact, the isoprenoid biosynthetic pathway was reported to be responsive to *E. necator* infection in leaves (Fung et al., 2008; Toth et al., 2016), which was not observed in berries. Additionally, metabolic profiling revealed an accumulation of long-chain fatty acids in infected berries (Figure 2.3); however, transcriptomic profiling of infected leaves suggested activation of fatty acid degradation pathways (Fung et al., 2008).

The combination of multiple omics may provide valuable evidence on grape berry defense mechanisms. In this study, it was shown that the transcriptional and metabolic reprogramming was more active at the green stage (Figure 2.3, Figure 2.5) when the infection levels tend to be higher. Expression of defense genes at the green stage declined at the *véraison* stage, including glutathione-s-transferases, phenylpropanoid biosynthesis genes, and PR-genes. This observation might be related to the fact that *E. necator* usually infects photosynthetic tissues, and ontogenic resistance has been observed both in susceptible berries and in older leaves in the transition from source to sink (Calonnec et al., 2018; Ficke et al., 2004, 2003; Gadoury et al., 2003). Moreover, activation of defense genes on infected leaves was shown to reach the maximum when most conidia penetrated the host cells and developed secondary hyphae; after that, the expression also declined (Fung et al., 2008).

2.4.1. Powdery mildew infection initiates a sequence of plant immunity-related responses in susceptible grape berries.

Supported by the enrichment analysis, overexpression of defense genes was observed, revealing an activation of defense responses (Figure 2.8) in both green and *véraison* susceptible grape berries. Pathogen infection is known to trigger innate immunity through PTI, i.e., through the recognition of pathogen-associated molecular patterns (Jones and Dangl, 2006). Pattern recognition receptors (PRR) coding genes were activated in infected berries at both stages,

including *BAK1*, *FLS2,* and *wall-associated kinases* (*WAK*) coding genes. Moreover, several other kinase receptors, including LRR-RKs (leucine-rich repeat receptor kinases) and Ser/Thr receptor-like kinases, were up-regulated upon infection, indicating an overall activation of the PTI. BAK1 is a leucine-rich repeat receptor-like protein kinase that forms a complex with FLS2 and with other LRR-RKs after ligand perception, activating downstream signaling responses (Chinchilla et al., 2007; Roux et al., 2011; Schulze et al., 2010). WAKs are receptor-like kinases that recognize both pectins from the cell wall and pectin fragments, oligogalacturonic acids (OGs), derived from pathogen action, activating defense responses through MAPK cascades (Brutus et al., 2010; Decreux and Messiaen, 2005; Kohorn and Kohorn, 2012). Clavata1 receptor kinase (CLV1) encoding genes were also up-regulated in response to PM infection at both berry developmental stages. Interestingly, several *CLV1* genes were down-regulated with the *Botrytis cinerea* infection (Agudelo-Romero et al., 2015), indicating a different grape response to biotrophs and necrotrophs.

Chitin, which is found in fungal cell walls, is perceived through plant LysM receptor like-kinases (LysM-RKs) initiating defense responses (Wan et al., 2008). One chitin elicitor-binding protein containing a LysM motif was responsive to PM infection at the *véraison* stage. *V. vinifera* LysM-RKs were shown to be involved in chitooligosaccharide-triggered immunity and in penetration resistance to *E. necator* (Brulé et al., 2019). Additionally, chitinases and three *β*-1,3-glucanases coding genes were up-regulated in response to PM infection at EL33 stage; however, two chitinases were down-regulated, suggesting that was an intent to suppress chitin-triggered signaling during PM infection. Suppression of chitin-triggered immunity was observed for cacao pathogens that express inactive chitinases during infection (Fiorin et al., 2018). These chitinases lack the catalytical activity but maintain the substrate binding capacity, preventing plant immunity by sequestering free chitin fragments (Fiorin et al., 2018).

Adapted pathogenic species are supposed to release effectors to suppress PTI. In turn, host plants activate a second layer of resistance, ETI, through the recognition of those effectors by R proteins inside host cells (Jones and Dangl, 2006). Activation of ETI can result in a hypersensitive response (HR) at the infection site. In this study, several genes coding R proteins, including NB-LRR proteins and EIX receptors, were up-regulated in response to PM, most of the cases at both stages. Several grapevine NBS-LRRs were identified to be related to the PM response (Goyal et al., 2019). Moreover, we detected several putative *E. necator* effector transcripts, including those coding for EKA-like proteins and CSEP, that might influence the host plant immunity. Most of the EKA-like protein transcripts were detected only at the green stage, suggesting that these effectors might be associated with earlier stages of infection.

Moreover, fungal pathogens can release hydrolytic enzymes that target the plant cuticle (cutinases, esterases, and lipases) and the cell wall (CAZymes) to facilitate the infection (reviewed by Ziv et al., 2018). In this study, *E. necator* transcripts coding for these hydrolytic enzymes were detected, supporting the successful infection of the Carignan grape berries.

Upon pathogen sensing and receptor activation, MAPK signaling cascades are initiated and act on WRKY transcription factors, which are regulators of defense responses to several pathogens (Eulgem and Somssich, 2007). Two MAPK and several WRKY genes were up-regulated in response to PM infection, at the green and both stages, respectively. Up-regulated *WRKY* genes included the *WRKY33* and *WRKY71*, which were shown to be involved in defense in grapevine, Arabidopsis, and rice (Liu et al., 2007; Merz et al., 2015; Zheng et al., 2006). Like observed in this work, WRKY genes were also induced in PM infected leaves of susceptible *V. vinifera* cv. Cabernet Sauvignon (Fung et al., 2008). Ectopic expression of *Vitis* WRKY genes was shown to increase resistance to PM (Li et al., 2010; Wang et al., 2017; Zhu et al., 2012).

Overall, results indicate that susceptible berries were able to activate the defenses in response to *E. necator* infection (Figure 2.8). Activation of defense-related genes and proteins was also observed on 'Cabernet Sauvignon' leaves upon PM infection, suggesting that PM-susceptible plants are able to initiate a basal defense; however, those responses are not enough to restrict fungal growth or to mitigate disease progression (Fekete et al., 2009; Fung et al., 2008; Marsh et al., 2010). Activated defense genes in grapevine leaves were shown to include *EDS1*, *MAPKK*, *WRKY*, *PR-1*, *PR-10*, and *STS* (Fung et al., 2008), similar to what was observed in this study, suggesting that susceptible 'Carignan' grape berries at early development stages initiate defense mechanisms similar to the ones observed in susceptible 'Cabernet Sauvignon' leaves. However, up-regulation of different paralogous genes between berries and leaves was observed in some cases. Moreover, isoprenoid biosynthetic pathway and fatty acid metabolism were differently affected by infection in berries and leaves (Fung et al., 2008; Toth et al., 2016), suggesting an organ-specific response in these pathways.

Despite the induction of defense strategies, 'Carignan' berries were susceptible to infection, which could be explained in part by the over-expression of *MLO* genes. MLO protein has seven transmembrane domains and C-terminal cytoplasmic calmodulin-binding domain and is known to be essential in the successful penetration of adapted PM species on several host plants (Kusch and Panstruga, 2017). In *V. vinifera*, three *VvMLOs* (*VvMLO3*, *VvMLO4*, and *VvMLO17*) were supposed to act as PM susceptibility genes (Feechan et al., 2008), and knockdown of *VvMLO7* together with *VvMLO6* resulted in reduced PM severity (Pessina et al., 2016). Calmodulin and calmodulin-like proteins are Ca^{2+} receptors involved in

Ca2+/calmodulin-mediated signaling. Here, calmodulin genes were up-regulated in response to PM infection. Calmodulin binding to barley MLO protein increased susceptibility to PM (Kim et al., 2002). Results suggest that 'Carignan' susceptibility to PM could be due at least partially to increased expression of *S (susceptible)*-genes. The presence of susceptibility genes on *V. vinifera* was observed in 'Chardonnay' where the QTL *Sen1 (Susceptibility to Erysiphe necator 1)* locus was identified as a source of susceptibility (Barba et al., 2014). Moreover, the activation of the defense mechanisms occurred possibly only upon infection and basal defenses might not be enough to fight the beginning of the infection. In the absence of the fungal infection, resistant grapevine plants were reported to have higher basal levels of SA and defense-associated transcript than susceptible plants (Fung et al., 2008).

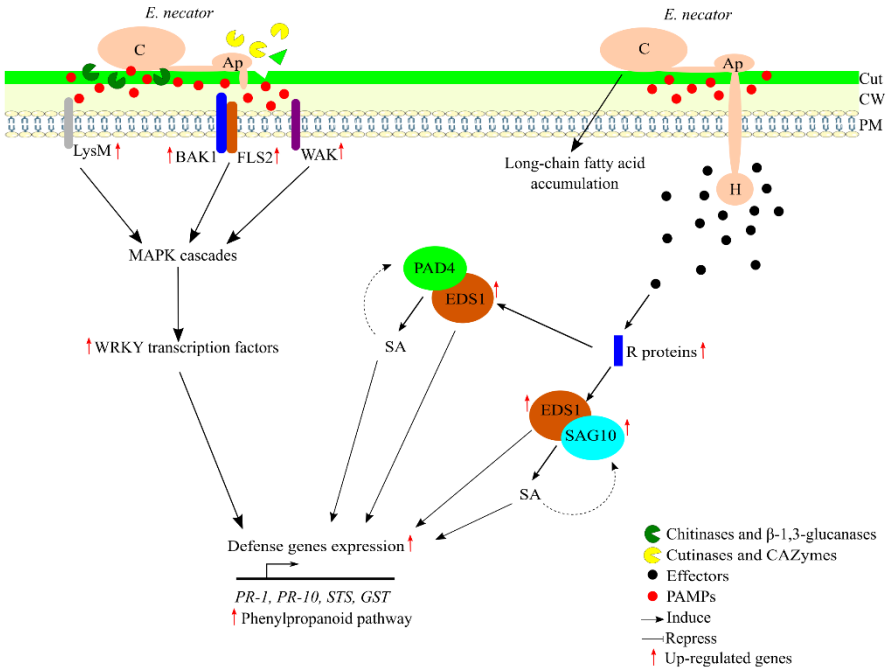

Figure 2.8 – Overview of defense responses during *Erysiphe necator* infection in grape berries. During powdery mildew infection, pattern recognition receptors (PRRs) recognize pathogen-associated molecular patterns (PAMPs) initiating PTI: MAPK cascades are activated acting on WRKY transcription factors that, in turn, regulate the expression of defense associated genes. Host cells release chitinases and *β*-1,3-glucanases that act on the fungal cell wall releasing chitin that is perceived by PRRs such as LysM receptor like-kinases. To suppress PTI, fungus release effectors which are then sensed by R proteins in the cytoplasm activating the ETI. This activation is facilitated by EDS1/PAD4 and EDS1/SAG101 complexes. Salicylic acid has a central role in defense against powdery mildew. *E. necator* infection also leads to the accumulation of long-chain fatty acids. C, conidia; Ap, appressorium; H, haustorium; Cut, cutin; CW, cell wall; PM, plasma membrane.

2.4.2. Response to powdery mildew infection is putatively regulated by the interaction of salicylic and jasmonic acids' metabolisms

Hormonal metabolism was strongly reprogrammed in response to PM infection, particularly, SA metabolism and signaling. SA is known to be a key regulator of response to biotrophic fungal infection, including powdery mildew (Fung et al., 2008). Hormonal

quantification revealed that free and conjugated SA levels increased in response to PM at both ripening stages, as observed in susceptible 'Cabernet Sauvignon' PM-infected leaves (Fung et al., 2008). EDS1 is a key regulator of innate immune responses against pathogens infection and interacts with two signaling partners, phytoalexin-deficient 4 (PAD4) and senescence-associated gene 101 (SAG101), regulating the SA signaling pathway (Feys et al., 2005; Wagner et al., 2013; Wiermer et al., 2005). In this study, both *EDS1* and *SAG101* genes were up-regulated in response to PM infection, especially at the *véraison* stage. Previous studies showed that *EDS1* promoter is induced by SA and *EDS1* levels in resistant *V. aestivalis* leaves were constitutively higher concerning the susceptible 'Cabernet Sauvignon', where infection induced the *EDS1* expression (Fung et al., 2008; Gao et al., 2014, 2010). In Arabidopsis, EDS1-PAD4 complex was found to be essential in basal resistance thought SA-mediated defenses (Rietz et al., 2011); however, the expression of the *PAD4* gene was not affected by PM infection, as observed in leaves after PM infection (Fung et al., 2008). The role of PAD4 in grapevine defense is poorly understood and the EDS1/PAD4 action might be more complicated than in Arabidopsis (Gao et al., 2014). Nevertheless, transcript levels might not reflect the involvement of PAD4 in grapevine defense and post-transcriptional regulation could be responsible for its activation upon infection. SA signaling regulates the expression of several defense-related genes, which were shown to be responsive to both PM infection and SA treatment (Toth et al., 2016).

JA signaling is classically associated with the response against necrotrophic pathogens; nevertheless, quantification of jasmonates suggested regulation of JA metabolism in response to *E. necator*. Despite the activation of genes related to JA biosynthesis (LOX and AOS), *cis*-OPDA and JA contents were not significantly affected by PM infection. On the other hand, JA-Glucoside was highly accumulated in infected berries, particularly at the green stage. These results suggest a redirection of JA to its conjugated form. Moreover, a tendency to increase in response to PM was observed for JA-Ile and OH-JA-Ile. JA-Ile is a bioactive jasmonate (Staswick and Tiryaki, 2004), and hydroxylation (OH-JA-Ile) is suggested to acts as a "stop" signal through catabolic inactivation of the JA-Ile (Koo et al., 2014, 2011). However, in a recent study, Jimenez-Aleman and co-workers (2019) demonstrated that 12-OH-JA-Ile maintained its bioactivity signals through the canonical JA-pathway and it might regulate specific jasmonate dependent responses. The involvement of JA signaling in biotrophs infection is still poorly understood, and this study suggests a modulation of jasmonates content in response to PM. Moreover, MeJA exogenous application on *V. vinifera* plants elicited tolerance to subsequent *E. necator* infection (Belhadj et al., 2006). Jasmonates might function as regulators of the SA-

mediated responses in a crosstalk manner (Robert-Seilaniantz et al., 2011). Nevertheless, the role of jasmonates in plant response to biotrophic fungus, as well as the regulation of SA and JA signaling pathways should be further explored.

Although the levels of IAA and ABA were not affected by PM infection, the expression of genes involved in metabolism and signaling were responsive to *E. necator*, such as IAA-amido synthetase, *PIN1*, *AUX1*, *NCED* and ABA-responsive protein, especially at the green stage. These results might indicate an involvement of IAA and ABA in PM infection response, independently of their accumulation. Recently, some auxins, except IAA (indole-3-acetic acid), were shown to be accumulated in response to PM in barley (Janeczko et al., 2019); therefore the action of less abundant auxins in response to PM in grapevine might be interesting to explore in the future.

2.4.3. Powdery mildew infection induced the reprogramming of berry metabolism and altered specific regulatory ripening processes

Transcriptional and metabolic data presented in this study suggest a reprogramming of several metabolic pathways in response to *E. necator*.

The first stage of infection is the contact of the pathogen with the plant cuticle, which is composed of cuticular wax and a matrix of cutin and protects the plant against abiotic stresses and pathogen penetration (Ziv et al., 2018). Accumulation of ursolic acid that is one of the main components of the fruit cuticular wax in some fruit plants (Lara et al., 2015), was reduced in infected berries at the EL35 stage. On the other hand, several long-chain saturated fatty acids, which are precursors for biosynthesis of cutin and wax, were accumulated in infected berries, with particular attention to eicosanoic acid (arachidic acid), and its derivative alcohol, and to docosanoic acid (behenic acid). Additionally, genes involved in fatty acid biosynthesis (acyl-CoA synthetase and omega-6 fatty acid desaturase) and in glycerophospholipids catabolism (triacylglycerol lipases) were up-regulated in response to infection. Powdery mildew was also previously reported to modify the fatty acid profile of fully developed infected berries and to induce the synthesis of eicosanoic acid (Petrovic et al., 2017). Several fatty acids from leaf and/or berry cuticular waxes of resistant genotypes were shown to have antifungal activity (Özer et al., 2017). Accumulation of long-chain fatty acids, as well as up-regulation of genes coding for acyl-CoA synthases, were also observed in ‘Trincadeira’ berries infected with *B. cinerea* (Agudelo-Romero et al., 2015), suggesting a role not only in response to biotrophic but also necrotrophic fungi. Activation of fatty acid biosynthesis and cuticle remodeling, as presented here, was also reported in tomato fruits infected with *C gloeosporioides* during

appressoria formation and before fungal penetration (Alkan et al., 2015). Curiously, the fatty acid biosynthesis was reported to be down-regulated in response to PM in leaves together with an up-regulation of genes related to fatty acid degradation (Fung et al., 2008).

Relative to nitrogen (N) metabolism, transcription analysis indicates an adjustment of N transport through the increased expression of nitrate and ammonium transporters. Supporting our results, Pike and co-worker (2014) observed an up-regulation upon PM infection of the nitrate transporter *VvNPF3.2* in susceptible 'Cabernet Sauvignon' grapevine and the *AtNPF3.1* in Arabidopsis, suggesting that the pathogen influences host metabolism in order to increase nitrate transport. Additionally, Jones and co-workers (2014) demonstrated that *E. necator* lacks some genes related to N metabolism., which might be associated with the obligate biotrophic lifestyle, supporting the activation of host genes related to the N transport.

Some genes encoding sugar and polyols transporters, as well as genes related to sugar metabolism, were up-regulated in response to infection, suggesting that the fungus induced the sugar transport. Nevertheless, the sugar content (fructose and glucose) was not changed in response to infection; therefore, sugars might be redirected to fungal consumption. Hexose transporter genes were also reported to be up-regulated in grape leaves upon *E. necator* infection (Hayes et al., 2010). Sugar transport was reported to be activated during powdery mildew infection in Arabidopsis as well (Fotopoulos et al., 2003). Additionally, several transcripts encoding sugar transporters were also detected in the fungus transcriptome. Enrichment analysis indicated that in infected berries the carbohydrate metabolism, specially the sucrose biosynthesis, was one of the categories enriched in *véraison*-responsive up-regulated genes; therefore, PM infection might have affected specific ripening processes mainly related to minor sugars, such as sucrose and polyols, maintaining the homeostasis of the main sugars (glucose and fructose) during ripening.

PM infection resulted in the activation of secondary metabolism through the up-regulation of genes from the phenylpropanoid pathway (flavonoid, stilbenoid, and lignin), resulting in the accumulation of phenylpropanoids, including catechins, resveratrol, and anthocyanins (Figure 2.6). Moreover, infections seem to deregulate the normal course of the phenylpropanoid pathway during ripening, anticipating its activation; particularly the accumulation of the anthocyanins, typical of the *véraison* stage, that started at the green stage. Stilbenes, such as resveratrol, function as phytoalexins and are accumulated in grapevine in response to stress (Jeandet et al., 2002). PM infection resulted in the up-regulation of several stilbene synthase (*STS*) genes, and consequently, in the accumulation of resveratrol in infected berries. *Trans*-resveratrol accumulation was also observed in PM-infected 'Sangiovese' red

grapes (Piermattei et al., 1999). *STS* genes and stilbenes were reported to be involved in response and resistance to PM in several *Vitis* species (Huang et al., 2016; Liu et al., 2019; Ma et al., 2018; Schnee et al., 2008; Xu et al., 2019; Yin et al., 2017), including the susceptible *V. vinifera* cv. Cabernet Sauvignon (Fung et al., 2008). Regarding another class of phytoalexins, two anthranilate N-benzoyltransferase (HCBT) coding genes involved in dianthramides biosynthetic pathway were over-expressed in infected berries.

2.5. Conclusions

Vitis vinifera varieties are highly susceptible to powdery mildew being 'Carignan' one of the most affected. Most studies on powdery mildew infection response were performed on grape leaves from greenhouse plants, which may not reproduce field conditions. This work is the first to integrates transcriptional, metabolic and hormonal profiling of 'Carignan' grape berries infected with PM. PM-susceptible grape berries were able to induce defense mechanisms (Figure 2.8) and accumulate defense-associated metabolites. However, activation of plant defenses was not enough to restrict fungal infection and to provide resistance. Induction of defense mechanisms was also observed in leaves from susceptible *V. vinifera* plants (Fung et al., 2008) but certain responses namely reprogramming of fatty acid metabolism and isoprenoid biosynthesis was different in grape berries, highlighting the importance of conduction studies in different grapevine organs. This work also provides several metabolites, such as resveratrol, gallic acid, eicosan-1-ol, eicosanoic, and docosanoic acids, that can be explored for posterior use as markers of infection at earlier ripening stages on field conditions. Results also suggests an involvement of JA signaling, normally associated with response to necrotrophic fungus.

2.6. Material and Methods

2.6.1. Sampling

Grape berry clusters were collected in 2017 from a commercial vineyard (*Vitis vinifera* L. cv. Carignan) at Torres Vedras region, Portugal (39º04'43.2''N, 9º20'58.9''W). Sampling was performed in two conditions, healthy berries and naturally infected berries, at two developmental stages: late green (EL33) and *véraison* (EL35). For each condition (PM infected and Control) and time point, four to five biological replicates were collected on 13 July and 2 August 2017 after visual inspection of symptoms. Grape clusters were harvested around 11.30 a.m. and immediately frozen in liquid nitrogen, transported in dry ice to the laboratory, and stored at -80 ºC until further use. Prior to transcriptomic and metabolomic analysis, berries were

deseeded and grounded to a fine powder. Three to four replicates were used for metabolomics and hormone quantification and some of those samples were pooled to obtain three independent biological replicates for RNA-seq analysis.

2.6.2. DNA extraction and biomass quantification

Combined berry and fungal DNA was extracted according to Lodhi et al. (1994) with some modifications. Before the RNase A purification, a treatment with 1/10 vol 2M KAc (1 h on ice) was added to the protocol to precipitate polysaccharides. Fungal biomass accumulation was relatively measured by real-time PCR (qPCR), according to Jones et al. (2014), by amplifying the *E. necator* elongation factor *EnEF1* (KHJ34692) and the grapevine actin *VvActin* as reference (Supplementary Table S2.5).

2.6.3. RNA extraction

RNA extraction was performed as described by Coelho et al. (2019). A DNase treatment was carried out using TURBO™ DNase according to the supplier's instructions (Invitrogen, USA). RNA was then purified using Spectrum™ Plant Total RNA kit (Sigma-Aldrich, USA).

2.6.4. RNA-seq and differential gene expression analysis

RNA-seq was performed in the Centre for Genomic Regulation (CRG, Barcelona, Spain). The corresponding cDNA libraries were prepared using the Illumina TruSeq Stranded mRNA Sample Prep kit v2 (ref. RS-122-2101/2) using 600 ng of total RNA. Poly(A)-mRNA selection using streptavidin-coated magnetic beads and subsequent RNA fragmentation to 300bp was performed. cDNA was synthesized using reverse transcriptase (SuperScript II, ref. 18064-014, Invitrogen) and random primers. The second strand of the cDNA incorporated dUTP in place of dTTP. Double-stranded DNA was further used for library preparation. dsDNA was subjected to A-tailing and ligation of the barcoded Truseq adapters. All purification steps were performed AMPure XP beads and eluted in 20 µl EB. Library amplification was performed by PCR on the size selected fragments using the primer cocktail supplied in the kit.

Final libraries were analyzed using Agilent DNA 1000 chip to estimate the quantity and check size distribution and were then quantified by qPCR using the KAPA Library Quantification Kit (ref. KK4835, KapaBiosystems) prior to amplification with Illumina's cBot. Library sequencing was performed on Illumina HiSeq2500 sequencer. Paired-end strand-specific reads of 75 nt were produced.

Reads alignment to the combined PN40024 12X.0 grapevine reference genome assembly and *E. necator* C-strain scaffolds (Jones et al., 2014) was performed using HISAT2 version

2.1.0 (Kim et al., 2015). Uniquely mapped reads were used for further analysis. The software package SAMtools (http://samtools.sourceforge.net/, (v. 1.3.1) was used for intermediary processing of the mapped reads (sam and bam files) including duplicates removal. The htseq-count tool (v.0.11.1) from HTSeq (Anders et al., 2015) was used to estimate unambiguous read count for each grapevine 12X V1 annotated transcript and *E. necator* transcripts. Normalization following the trimmed mean of M-values (TMM) method (Robinson and Oshlack, 2010) and differential expressed genes (DEGs) search were performed in edgeR v.3.24.3 (Robinson et al., 2010). Reads per kb of exon per million fragments mapped (RPKM) were calculated using edgeR. Read count data was used for the Multidimensional Scaling (MDS) plot. DEGs were selected considering the false discovery rate (FDR) of ≤ 0.05 and fold change of ≥ 2.0 or ≤ –2.0.

2.6.5. Functional analysis of differentially expressed genes

The list of DEGs resulting from the RNA-seq analysis was analyzed using FatiGO (Al-Shahrour et al., 2007) to identify functional categories significantly enriched according to the classification of 12X V1 annotation (Grimplet et al., 2012). FatiGO uses Fisher's exact test to compare each DEGs list with the list of total non-redundant transcripts housed in the grapevine 12X V1 gene predictions (Grimplet et al., 2012) and significantly enriched categories were selected considering the corrected p-value ≤ 0.05 (Benjamini & Hochberg correction for multiple testing).

2.6.6. Real-time PCR

First-strand cDNA was synthesized from 2 µg of total RNA, according to Fortes et al. (2011). Real-time PCRs (qPCRs) were carried out using the StepOne™ Real-Time PCR System (Applied Biosystems, USA). Cycling conditions were 95 °C for 10 min, followed by 42 cycles of 95 °C for 15 s and primers' annealing temperature for 1 min. Relative expression data were obtained from 3-4 biological replicates and duplicate technical replicates (in separate plates). The standard curve was built using a serial dilution of mixtures of all cDNAs analyzed (1:1, 1:4, 1:16, 1:64, and 1:256), and used to check primers efficiency. Data were normalized using the expression curves of the actin gene (VIT_04s0044g00580) and elongation factor 1α gene (VIT_06s0004g03220). All primers used are shown in Supplementary Table S2.5.

2.6.7. Soluble metabolites

The profiling of soluble metabolites was performed by gas chromatography coupled to electron impact ionization time-of-flight mass spectrometry (GC-EI/TOF-MS), as specified by Dethloff et al. (2014).

Soluble metabolites were extracted, as previously described by Agudelo-Romero et al. (2013), from 300 ± 30 mg (fresh weight) of deep-frozen powder by 1 mL ethylacetate for 2 h agitation at 30 °C. Extracts were centrifuged for 5 min at 14000 rpm, and two aliquots of 300 µL from the ethylacetate fraction were dried by vacuum concentration and stored at -20 °C.

Chemical derivatization and retention index calibration were performed prior to injection, as described by Dethloff et al. (2014). GC-EI/TOF-MS analysis was performed using an Agilent 6890N24 gas chromatograph (Agilent Technologies, Germany) connected to a Pegasus III time-of-flight mass spectrometer (LECO Instrumente GmbH, Germany), with splitless injection onto a Varian FactorFour capillary column (VF-5 ms) of 30 m length, 0.25 mm inner diameter, and 0.25 mm film thickness (Varian-Agilent Technologies, Germany). Chromatograms were acquired, visually controlled, baseline corrected and exported in NetCDF file format using ChromaTOF software (Version 4.22; LECO, St. Joseph, USA).

Compounds were identified by mass spectra and retention time index matching to the reference collection of the Golm Metabolome Database (Hummel et al., 2010; Kopka et al., 2005) with manual supervision using TagFinder software (Luedemann et al., 2008). Guidelines for manually supervised metabolite identification were the presence of at least three specific mass fragments per compound and a retention index deviation of less than 1.0 % (Strehmel et al., 2008). Metabolite intensities were normalized by sample fresh weight and internal standard (C_{22}), maximum scaled, and $\log_2$-transformed to approximate normal distribution for statistical analysis.

2.6.8. Volatile metabolites

Volatile profiling used 500 ± 50 mg (fresh weight) of deep-frozen grape berry powder and was performed by solid-phase micro-extraction (SPME) and GC coupled to an EI/quadrupole MS (GC-EI/QUAD-MS) using Agilent 5975B VL GC-MSD system and a StableFlex^{TM} SPME-fiber with 65 µm polydimethylsiloxane/divinylbenzene (PDMS-DVB) coating (Supelco, USA) as described by Vallarino et al. (2018). SPME samples were taken from the headspace with 10 min incubation at 45 °C, 5 min adsorption at 45 °C and 1 min desorption at 250 °C onto a DB-624 capillary column with 60 m length, 0.25 mm inner diameter, and 1.40 µm film thickness (Agilent Technologies, Germany). GC temperature programming was 2 min

isothermal at 40°C followed by a 10 °C/ min ramp to 260 °C final temperature, which was held constant for 10 min. The Agilent 5975B VL GC-MSD system was operated with a constant flow of helium at 1.0 mL/ min. Desorption from the SPME fiber was at 16.6 psi with an initial 0.1 min pulsed-pressure at 25 psi. The subsequent purge was 1 min at a purge flow of 12.4 mL/ min. System stability was controlled and the sample sequence randomized. GC-EI/QUAD-MS chromatograms were acquired with mass range set to 30–300 m/z and a 20 Hz scan rate. Chromatograms were acquired, visually controlled, and exported in NetCDF file format using Agilent ChemStation-Software (Agilent) and baseline-corrected with MetAlign software (Lommen, 2009).

Compounds were identified as described above using TagFinder software (Luedemann et al., 2008) by mass spectra and retention time matching to the reference collection of the GMD for volatile compounds (Hummel et al., 2010; Kopka et al., 2005). Guidelines for manually supervised identification were the presence of at least three specific mass fragments per compound and a retention time deviation of less than 3 %. Metabolite intensities were normalized by sample fresh weight, maximum scaled, and $\log_2$-transformed to approximate normal distribution for statistical analysis.

2.6.9. Hormonal profiling

About 30 mg of dry berry tissue were extracted and homogenized in 1.5 ml methanol containing 60 ng D4-SA (Santa Cruz Biotechnology, USA), 60 ng D6-JA (HPC Standards GmbH, Germany), 60 ng D6-ABA (Santa Cruz Biotechnology, USA), and 12 ng D6-JA-Ile (HPC Standards GmbH) as internal standards. Samples were agitated on a horizontal shaker at room temperature for 10 min. The homogenate was mixed for 30 min and centrifuged at 13,000 rpm for 20 min at 4 °C and the supernatant was collected. The homogenate was re-extracted with 500µl methanol, mixed and centrifuged and the supernatants were pooled. The combined extracts were evaporated under reduced pressure at 30 °C and dissolved in 500µl methanol.

Phytohormone analysis was performed by LC-MS/MS as in Heyer et al. (2018) on an Agilent 1260 series HPLC system (Agilent Technologies) coupled to a tandem mass spectrometer API5000 (SCIEX, Darmstadt, Germany). Since we observed that both, the D6-labeled JA and D6-labeled JA-Ile standards (HPC Standards GmbH, Cunnersdorf, Germany) contained 40 % of the corresponding D5-labeled compounds, the sum of the peak areas of D5- and D6-compound was used for quantification. Details of the instrument parameters and response factors for quantification can be found in Supplementary Table S2.7.

Indolacetic acid was quantified using the same LC-MS/MS system with the same chromatographic conditions but using positive mode ionization with an ion spray voltage at 5500 eV. Multiple reaction monitoring (MRM) was used to monitor analyte parent ion → product ion fragmentations as follows: m/z 176 →130 (collision energy (CE) 19 V; declustering potential (DP) 31 V) for indolacetic acid (IAA); m/z 181 →133 + m/z 181 →134 + m/z 181 →135 (CE 19 V; DP 31 V) for D5-indolacetic acid.

2.6.10. Anthocyanin and total phenolic content quantification

Anthocyanin quantification was measured as described previously (Agudelo-Romero et al., 2013). Total relative anthocyanin concentration was expressed as absorbance value at 520nm per g of freeze-dried weight.

Total phenolic content was measured using the Folin-Ciocalteau colorimetry method using a gallic acid calibration curve ranging from 12.5 to 125 µg mL^{-1} (Singleton and Rossi, 1965). Phenolics were extracted from 50 mg of lyophilized grape berry samples in 1.5 mL of ultra-pure water and centrifuged at 13000 rpm for 30 min. Twenty µL of the diluted extract (1:10) was added to 1 ml of diluted Folin-Ciocalteu reagent (1:10). Upon 10 min incubation, 800 µL of a 7.5% (w/v) sodium carbonate was added to the reaction, incubated for 30 min incubation. Absorbance was measured at 743 nm. Total phenolic content was expressed as gallic acid equivalents (GAE, mg) per mg freeze-dried weight.

2.6.11. Protein extraction and phenylalanine ammonia lyase (PAL) enzymatic assay

Protein extraction from grape berry powder was performed as described by Conde et al. (2016), with minor alterations. Briefly, 1 g of deep-frozen grape berry powder was mixed with 2 mL of extraction buffer and centrifuged at 18000xg for 20 min at 4ºC. Supernatant was used for further assays. Protein extraction buffer contained 50 mM Tris-HCl pH 8.8, 5 mM $MgCl_2$, 1 mM EDTA pH, 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM dithiothreitol (DTT) and 1% (w/v) PVPP. Total protein content of each extract was determined by the Bradford assay (Bradford, 1976), using bovine serum albumin as standard.

Phenylalanine ammonia lyase enzymatic activity was measured in a reaction mixture containing 200 µL of protein extract, 500 µL 250mM Tris-HCl pH 8.8, and 250 µL substrate (40 mM L-phenylalanine, 100 mM Tris-HCl pH 8.8) and was incubated at 37 C for 30 min. The rate of conversion of L-phenylalanine to cinnamic acid was monitored spectrophotometrically at 290nm each 10 min (ε = 17400 M^{-1} cm^{-1}). Reaction was initiated by the addition of L-phenylalanine.

2.6.12. Statistical analysis

Statistical analysis of metabolomics data was performed using log_2-transformed response ratios and included Student's *t*-test, one- and two-way ANOVA, Kruskal–Wallis and Wilcoxon rank-sum tests. For multiple comparisons, the Benjamini & Hochberg correction was used which defines a sequential *p*-value procedure that controls the expected proportion of falsely rejected hypotheses - the false discovery rate (FDR). Principal component analysis was performed applying the MetaGeneAlyse web application (v.1.7.1; http://metagenealyse.mpimp-golm.mpg.de) and the R function prcomp to the log_2-transformed response ratios with missing value substitution, $log_2 = 0$. Heatmaps were designed using the R package ComplexHeatmap (Gu et al., 2016). Venn diagrams were designed using Venny 2.1 web application (v. 2.1.0; http://bioinfogp.cnb.csic.es/tools/venny/).

2.7. Supplementary Data

Supplementary Figure S2.1 – *Erysiphe necator* biomass accumulation estimated by qPCR in powdery mildew (PM) infected and control grape berries at green (EL33) and *véraison* (EL35) stages. Bars and whiskers represent averages and standard deviation (SD).

Supplementary Figure S2.2 – Principal component analysis (PCA) of metabolic profiles of grape berries at EL33 and EL35 stages with (PM Infected) and without (Control) powdery mildew infection.

Supplementary Figure S2.3 – Venn diagrams of differentially expressed genes. (A) Infection-responsive transcripts in EL33 and EL35 stages. (B) *Véraison*-responsive transcripts in control and PM-infected berries.

Supplementary Figure S2.4 – Multidimensional scaling (MDS) of RNAseq analysis of grape berries at EL33 and EL35 stages with (PM Infected) and without (Control) powdery mildew (PM) infection.

Supplementary Figure S2.5 - Enriched functional subcategories (adjusted p-value ≤ 0.05) in response to development (EL35 vs EL33). Circles' size represents the number of genes (log10) for each functional subcategory. Complete dataset in Supplementary Table S2.3.

Supplementary Figure S2.6 – Real-time PCR validation of the expression of thirteen genes in control and powdery mildew infected berries at green (EL33) and *véraison* (EL35) stages.

Supplementary Figure S2.7 – Pearson correlation between the expression fold-changes (log_2) obtain with real-time PCR and RNA-seq data.

Supplementary Figure S2.8 – Total phenolic content and phenylalanine ammonia-lyase (PAL) enzyme assay. Bars and whiskers represent averages and standard deviation (SD). GAE, gallic acid equivalents.

Supplementary Table S2.1 – Normalized responses of profiled volatile and polar metabolites from developing berries of *Vitis vinifera* cv. Carignan non-infected (control) and infected with powdery mildew.

Supplementary Table S2.2 – List of *Vitis vinifera* genes differentially expressed comparing infected and control berries at EL33 and EL35 and comparing development stages (EL35 vs. EL33).

Supplementary Table S2.3 – Functional enrichment analysis of differentially expressed transcripts in response (A) to PM infection and (B) to *véraison* (EL35) stage. Functional categories significantly over-represented (0.05 Benjamini-Hochberg adjusted *p*-value in a Fisher's exact test) within each expression profile. Functional classification from Grimplet et al., BMC Res Notes 2012.

Supplementary Table S2.4 – List of *Erysiphe necator* predicted genes in infected berries at green (EL33) and *véraison* (EL35) stages.

Supplementary Table S2.5 – List of primers used in real-time PCR.

Supplementary Table S2.6 – Summary of RNA-seq sequencing and mapping statistics.

2.8. References

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Chapter 3

The *LATERAL ORGAN BOUNDARIES domain* gene family in grapevine: genome-wide characterization and expression analyses during developmental processes and stress responses

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The work present in this chapter has been published, with minor changes:

Grimplet, J.*, **Pimentel, D**.*, Agudelo-Romero, P., Martinez-Zapater, J.M, and Fortes, A.M. (2017) The *LATERAL ORGAN BOUNDARIES Domain* gene family in grapevine: genome-wide characterization and expression analyses during developmental processes and stress responses. Scientific Reports 7: 15968. doi: 10.1038/s41598-017-16240-5.

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Authors' Contributions: Ana Margarida Fortes and Jerome Grimplet designed the study. J.G., Patricia Agudelo-Romero, Diana Pimentel, and A.M.F. analyzed the data. D.P. and A.M.F. wrote the manuscript with valuable input from J.G. and José Martinez-Zapater. All the authors revised and approved the manuscript.

3.1. Abstract

LATERAL ORGAN BOUNDARIES (LOB) DOMAIN (LBD) constitute a family of plant-specific transcription factors with key roles in the regulation of plant organ development, pollen development, plant regeneration, pathogen response, and anthocyanin and nitrogen metabolisms. However, the role of LBDs in fruit ripening and in grapevine (*Vitis vinifera* L.) development and stress responses is poorly documented. By performing a model curation of *LBDs* in the latest genome annotation 50 genes were identified. Phylogenetic analysis showed that *LBD* genes can be grouped into two classes mapping on 16 out of the 19 *V. vinifera* chromosomes. New gene subclasses were identified that have not been characterized in other species. Segmental and tandem duplications contributed significantly to the expansion and evolution of the *LBD* gene family in grapevine as noticed for other species. The analysis of *cis*-regulatory elements and transcription factor binding sites in the *VviLBD* promoter regions suggests the involvement of several hormones in the regulation of *LBDs* expression. Expression profiling suggests the involvement of LBD transcription factors in grapevine development, berry ripening and stress responses. Altogether this study provides valuable information and robust candidate genes for future functional analysis aiming to clarify mechanisms responsible for the onset of fruit ripening and fruit defense strategies.

3.2. Introduction

Transcription factors play an important role in the regulation of plant development and disease response. Among them, LATERAL ORGAN BOUNDARIES DOMAIN (LBD) proteins defined by a conserved N-terminal LATERAL ORGAN BOUNDARIES (LOB) domain is a family of plant-specific transcription factors with key roles in the regulation of plant organ development (Majer and Hochholdinge, 2011; Xu et al., 2016). The heterodimeric interactions between the Arabidopsis AS1, AS2, and JLO proteins are involved in the establishment of organ boundaries (Rast and Simon, 2012). AS2 (LBD6) interacts with AS1 in the process of leaf formation and both are known to be required for repression of meristematic genes and establishment of leaf adaxial-abaxial polarity (Semiarti et al., 2001). These proteins are also involved in the development of sepal and petal primordia of flowers by repressing boundary-specifying genes for normal development of the organ (Xu et al., 2008). JLO/LBD30 is a general regulator of cell specification and organ patterning throughout plant development (Borghi et al., 2007). On the other hand, LBD16, LBD18, and LBD29 regulate lateral root

organogenesis in Arabidopsis as direct targets of Aux/IAA–ARF modules in the auxin signaling pathway (Okushima et al., 2007).

Recent studies showed that these proteins are also involved in pollen development, plant regeneration, photomorphogenesis, pathogen response, and anthocyanin and nitrogen metabolisms (Xu et al., 2016). In this way, LBD27/SIDECAR POLLEN (SCP) and LBD10 are Arabidopsis microspore-specific LBD proteins having cooperative and unique roles in male gametophyte development (Kim et al., 2015). The Arabidopsis LBD proteins LBD16, LBD17, LBD18, and LBD29 are key regulators in the callus induction process associated with plant regeneration and establish a molecular link between auxin signaling and the plant regeneration program (Fan et al., 2012). Arabidopsis *LBD20* is a *Fusarium oxysporum* susceptibility gene that appears to regulate components of jasmonic acid (JA) signaling required for full elicitation of *F. oxysporum*- and JA-dependent responses (Thatcher et al., 2012b). Arabidopsis LBD25/DDA1 is involved in the regulation of light/dark-dependent hypocotyl elongation (Mangeon et al., 2011). LBD proteins have also been involved in developmental processes in non-model plants such as secondary phloem growth in *Populus* (Yordanov et al., 2010) and pulvinus differentiation and petiole development in legumes (Chen et al., 2012).

The characteristic LOB domain comprises a C-block containing four cysteines with spacing (CX2CX6CX3C) required for DNA-binding activity, a Gly-Ala-Ser (GAS) block and a leucine zipper-like coiled-coil motif (LX6LX3LX6L) responsible for protein dimerization (Majer and Hochholdinge, 2011; Xu et al., 2016). Several LBD proteins are capable to form homo- and hetero-dimers (Kim et al., 2015; Majer et al., 2012; Rast and Simon, 2012; Xu et al., 2016). Recently, it was demonstrated that the conserved proline residue in the GAS block is also crucial for the DNA-binding activity of Arabidopsis LBD16 and LBD18 proteins which have a role in lateral root formation (Lee et al., 2013b). The LBD gene family can be divided into two classes according to the structure of the LOB domain (Iwakawa et al., 2002; Shuai et al., 2002). Class I *LBD* genes contain a perfectly conserved CX2CX6CX3C zinc finger-like domain and an LX6LX3LX6L leucine zipper-like coiled-coil motif, whereas class II *LBD* genes only have a conserved zinc finger-like domain (Shuai et al., 2002). The majority of *LBD* genes belong to class I. Class II LBD proteins have an incomplete, probably not functional, leucine zipper that cannot form a coiled-coil structure (Majer and Hochholdinge, 2011).

Functional analysis, mainly in Arabidopsis, rice and maize, revealed that class I *LBD* genes are mostly involved in plant development such as lateral organ (leaf and flower) development (Majer and Hochholdinge, 2011; Xu et al., 2016), and in auxin signal transduction cascade that leads to the formation of lateral roots (Feng et al., 2012; Lee et al., 2015; Liu et al.,

2005; Okushima et al., 2007). By contrast, class II genes seem to be involved in metabolism, particularly as repressors of anthocyanin synthesis and N availability signals in the plant (Rubin et al., 2009; Scheible et al., 2004).

LOB domain proteins are suggested to act as transcription factors based on their nuclear localization (Lee et al., 2009; Okushima et al., 2007), and their capacity to bind to DNA motifs (Husbands et al., 2007). The DNA-binding affinity of ASL4 (LOB) was reduced by interacting with bHLH048 proteins (Husbands et al., 2007). The variable C-terminal region of LBD proteins confers transcriptional control on downstream gene expression (Majer et al., 2012).

In silico genome analyses predicted the presence of 43 *LBD* members in *Arabidopsis thaliana* and *Zea mays*, 35 in *Oryza sativum* and 58 in *Malus domestica* (Schnable et al., 2009; Shuai et al., 2002; Wang et al., 2013; Yang et al., 2006). As more species have their complete reference genome sequenced, additional *LBD* genes can be identified, and the biological roles of this poorly studied gene family clarified.

Grapevine has been a widely-studied species during the last decade at the genomics level. The release of the whole grapevine genome sequence in 2007 represented a breakthrough to promote its molecular genetics analysis (Jaillon et al., 2007). Based on the published sequence data, a comprehensive analysis of a given gene family can be performed to uncover its molecular functions, evolution and gene expression profiles. These analyses can contribute to the understanding of how genes in gene families control traits at a genome-wide level.

Recent preliminary analyses predicted 40 *LBD* genes in the grapevine genome (Cao et al., 2016) using an older version of the grapevine genome and without manual curation. In this work, we identified 50 *LBD* genes and performed a detailed structural analysis and mapping of these genes on the grapevine chromosomes. This gene family has been compared with similar families in thirty-three plant species. Finally, identification of c*is*-acting regulatory elements in promoter regions together with expression analyses based on microarray and RNAseq data suggest that LBD proteins are involved in the process of grape ripening and in the plant response to abiotic and biotic stresses.

3.3. Results

3.3.1. Structural annotation of LBD genes, phylogenetic analysis, and nomenclature.

Genes that were previously identified as *LATERAL ORGAN BOUNDARIES DOMAIN* in the grapevine genome (Grimplet et al., 2012) were used to performed sequence comparison analyses with BLASTX, either against the most up to date gene predictions from CRIBI V1 and V2, the NCBI refseq (remapped on the 12Xv2 of the genome assembly) and the VCOST (on the 12Xv2 of the genome assembly). Analyses were also performed directly against the reference genome sequence with TBLASTX to check whether any potential gene could have been missed by these predictions. By using these approaches, we identified 50 genome regions that shared homology with at least one of the genes.

Gene models were curated using the data collected from gene structure comparisons using different databases as well as the available inflorescence and flower RNAseq data from the laboratory (data not shown). RNAseq data allowed to evaluate whether newly detected genes, not represented in microarray data, showed expression, by redoing the bioinformatics analysis of original RNAseq data with an updated GFF file. A total of 50 *LBD* genes having a putatively functional structure were identified in the grapevine genome (Table 3.1), which is similar to the number of genes identified in the Arabidopsis genome (43 genes) (Iwakawa et al., 2002; Shuai et al., 2002). Data relative to the detection of *LBD* genes in previous genome annotations or gene-sets are summarized in Supplementary Table S3.1. The majority of the genes were identified in all the annotations. However, four genes were not detected in the automatic annotation CRIBIv1, three were not detected in the CRIBIv2, six were missing in the VIB annotation, and two in the NCBI refseq annotation. Representative sequences for each gene model were selected from the different annotations based on their quality (apparently full-length gene when compared to other species, no chimera): 13 were selected from the CRIBI, 2 from the VIB annotation and the remaining 35 from the refseq annotation. These genes are integrated in the Grapevine annotation V3 recently published (Canaguier et al., 2017).

Table 3.1. Genome localization of the 50 grapevine *VviLBD* genes.

Locus ID	Short Name	Strand	Position	Locus ID	Short Name	Strand	Position
Vitvi08g00144	LBDIa1 LBD20	+	2648151 - 2649186	Vitvi13g00551	LBDIf5	-	5061981 - 5063306
Vitvi15g00736	LBDIa2 LBD19	+	14992229 - 14994929	Vitvi13g00552	LBDIf6	-	5073630 - 5074839
Vitvi15g00735	LBDIa3	-	14983081 - 14985200	Vitvi13g00549	LBDIf7	+	5039284 - 5040392
Vitvi07g00573	LBDIa4 LBD16	-	6228524 - 6229895	Vitvi13g00545	LBDIf8	+	4985620 - 4986382
Vitvi13g00333	LBDIa5 LBD33	+	3457062 - 3457909	Vitvi13g00546	LBDIf9	+	5011144 - 5011863
Vitvi07g00572	LBDIa6	+	6220003 - 6220810	Vitvi13g00556	LBDIf10	-	5144789 - 5155693
Vitvi17g00890	LBDIc1 LOB	-	10710469 - 10712731	Vitvi13g00543	LBDIf11	+	4954628 - 4956343
Vitvi14g01707	LBDIc2	+	27155871 - 27157919	Vitvi06g00336	LBDIf12	-	4180846 - 4182157
Vitvi13g00085	LBDIc3 LBD21	-	817035 - 817745	Vitvi06g00338	LBDIf13	-	4201430 - 4202390
Vitvi00g00480	LBDIc4 LBD6	+	11326893 - 11330106	Vitvi16g01446	LBDIg1	+	17405415 - 17406086
Vitvi00g01060	LBDIc5	-	22493178 - 22495116	Vitvi15g01216	LBDIi1	+	17253530 - 17254344
Vitvi07g01328	LBDIc6	-	18720228 - 18722222	Vitvi15g01217	LBDIi2	+	17259993 - 17261012
Vitvi07g01326	LBDIc7	-	18699491 - 18702938	Vitvi04g01768	LBDIi3	+	996279 - 997019
Vitvi07g01327	LBDIc8	-	18708083 - 18709964	Vitvi14g01878	LBDIi4 LBD27	-	28646091- 28646927
Vitvi16g00859	LBDIc9	-	15931002 - 15932161	Vitvi17g00520	LBDIi5	-	6117662 - 6119015
Vitvi19g01589	LBDId1 LBD3	+	21536622 - 21539654	Vitvi09g00188	LBDIi6 LBD22	-	2066342 - 2067873
Vitvi10g01237	LBDId2 LBD4	-	17047348 - 17048794	Vitvi12g00230	LBDIi7 LBD2	-	3392281 - 3394534
Vitvi06g00706	LBDId3	+	7971884 - 7972497	Vitvi11g00169	LBDIi8	+	1720665 - 1722595
Vitvi13g00109	LBDId4	+	1022072 - 1022675	Vitvi14g01193	LBDIIa1	+	21211055 - 21212346
Vitvi13g00144	LBDId5	-	1309999 - 1311255	Vitvi17g00325	LBDIIa2	-	3791838 - 3793171
Vitvi06g00772	LBDId6 LBD13	-	8584220 - 8586134	Vitvi01g00291	LBDIIa3	-	3210258 - 3211492
Vitvi13g01866	LBDIf1	-	5100511 - 5101131	Vitvi01g00290	LBDIIa4	+	3204504 - 3205665
Vitvi13g01867	LBDIf2	-	5102158 - 5103523	Vitvi18g00677	LBDIIb1	+	7746353 - 7747276
Vitvi13g00555	LBDIf3	-	5130143 - 5136580	Vitvi07g01610	LBDIIb2 LBD39	+	21897655 - 21899042
Vitvi13g00559	LBDIf4	-	5173575 - 5179644	Vitvi03g00628	LBDIIc1	-	7098961 - 7099834

Regarding nomenclature, a phylogenetic tree of the LBD protein-coding genes in *V. vinifera* and Arabidopsis was constructed (Figure 3.1) as suggested by the Super-Nomenclature Committee for Grape Gene Annotation (sNCGGa) (Grimplet et al., 2014). A bootstrap value of 70, as recommended by the Committee, allowed to discriminate the genes within the majority of the classes but for some of them the phylogenetic analysis was complemented by motif analysis to detect conservation within classes and determine the affiliation of the genes inside

some classes. The use of lower bootstrap values allowed to retrieve the same classes as in Arabidopsis. Class Id is the only family where the genes are not all within the same branch. The genes are part of a subtree with the class If, but all the genes clustered with an Arabidopsis gene from class Id with a bootstrap value higher than 70. Class Ic is hardly conserved with a bootstrap of 28 necessary to maintain the tree architecture. However, the clear consensus is found in the GAS motif and all the genes clustered with an Arabidopsis gene from class Ic with a bootstrap value higher than 70. Class IIb requires a bootstrap of 54 to maintain the tree architecture but also clear conservation is observed in the LX6LX3LX6 motif. Class Ii requires a bootstrap of 7 to maintain the tree architecture, which is rather low, and four genes were not clustering with an Arabidopsis gene from class Ic with a bootstrap value higher than 70. As in other species, *VviLBD* genes fall into two classes: Class I with 43 genes and Class II with 7 genes, relative to 37 and six in Arabidopsis (Iwakawa et al., 2002; Shuai et al., 2002). Class I *VviLBD* genes were grouped into six subclasses (a, c, d, f, g, and i) and Class II genes into three subclasses (a–c). Arabidopsis *LBD* genes were not clustered in subclass IIc, which includes only the *VviLBDIIc1* gene. Only two Arabidopsis *LBD* genes (*LBD1* and *LBD11*) were grouped in subclass If with thirteen *VviLBDIf (1–13)* genes. For individual gene nomenclature, since both Arabidopsis nomenclature and previously named *Vitis* genes were named based on a poorly informative numeric code and few clear orthologs were identified, gene symbols/names were adapted to the class, the subclass and a distinctive number as proposed for *Vitis* genes nomenclature (Grimplet et al., 2014). Correspondences among different nomenclatures are described in Supplementary Table S3.1.

Regarding the exon/intron structure (Supplementary Figure S3.1), the majority of the *VviLBD* genes presented two exons (37 genes), as it is commonly observed in other plant species (Luo et al., 2016; Wang et al., 2013; Yang et al., 2016, 2006). Nevertheless, four of them have a non-coding exon (*LBDIc1 LOB*, *LBDIc6*, *LBDIc8*, *LBDIi1*), while *LBDIc6* expression was not detected according to RNAseq data. Thirteen genes present a different exon/intron structure comparing to the other 37 genes: five of them did not have any intron (*LBDIc2*, *LBDIc3*, *LBDIc9*, *LBDIi3*, and *LBDIi4)*, and seven of them contained three exons (*LBDIc5*, *LBDId5*, *LBDId6*, *LBDIf3*, *LBDIf4*, *LBDIi7*, and *LBDIi8*). However, *LBDIc5* presented two non-sense exons. Finally, *LBDIc4* presented five exons, although four of them were predicted as non-sense. Four of the five genes with predicted non-sense exons belong to Class Ic. The size of the *LBD* gene locus varied ten times, ranging from 603 nucleotides (*VviLBDId4*) to 6437 nucleotides (*VviLBDIf3*).

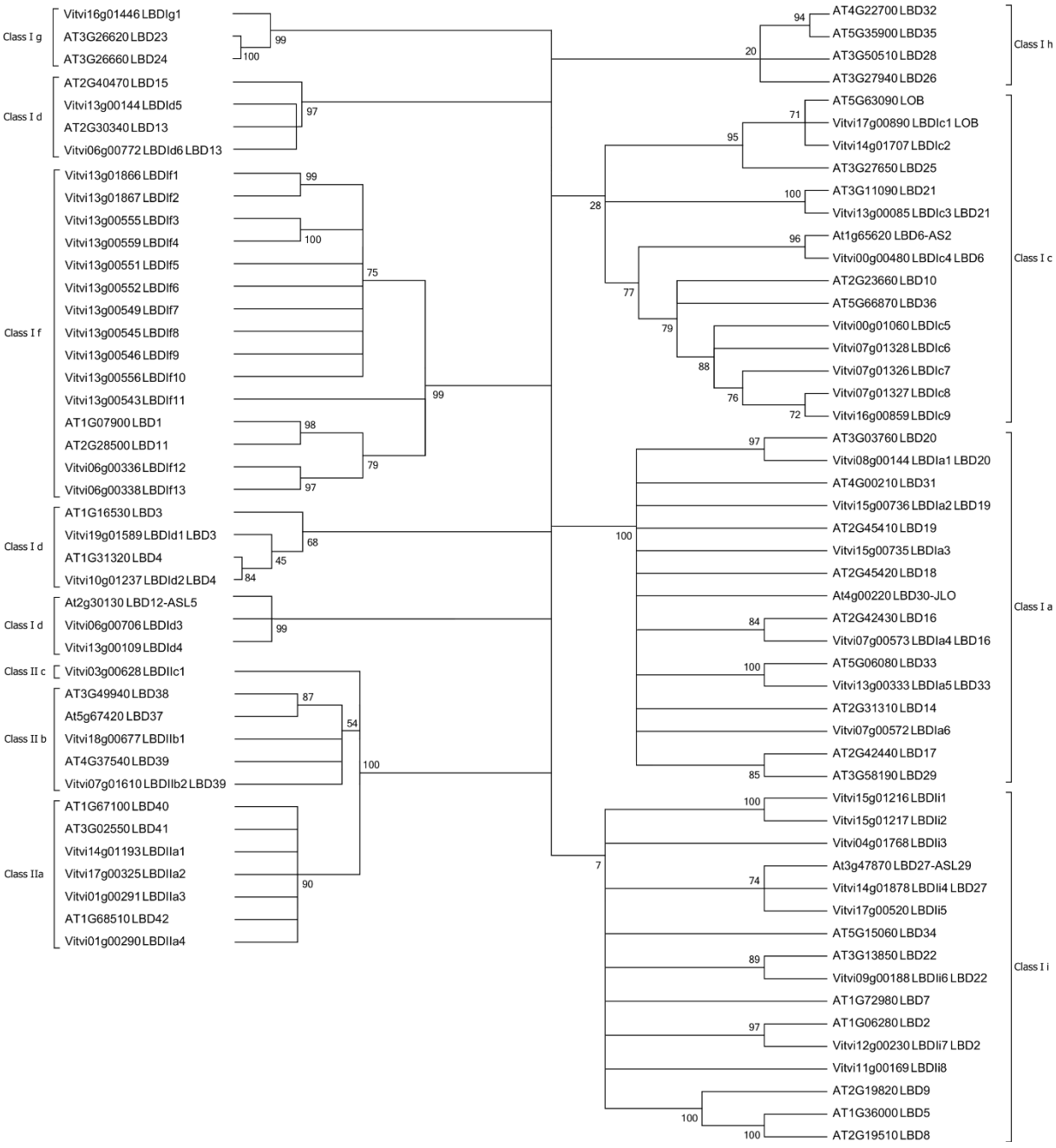

Figure 3.1. Phylogenetic analysis of grapevine and Arabidopsis *LBD* genes. Two classes were identified, Class I subdivided into six subclasses (**a**,**c**,**d**,**f**,**g**, and **i**) and Class II into three (**a**–**c**).

3.3.2. Motif analyses and orthologous relationships.

The LBD transcription factor family has a conserved LOB domain in the N terminus that comprises a C-block, a GAS block, and a leucine-zipper-like coiled-coil motif (Iwakawa et al., 2002; Shuai et al., 2002). Multiple sequence alignment within all of the VviLBD predicted proteins showed that the CX2CX6CX3C zinc finger-like domain was conserved in all 50 predicted protein sequences (Figure 3.2, Supplementary Figure S3.2). In addition, VviLBD proteins had a completely conserved G amino acid at the GAS block (Figure 3.2). Class I LBD

proteins presented a phenylalanine (F) and a histidine (H) completely conserved at the FX2(V/A)H motif, which represents the beginning of the GAS block. At the DP(V/I) YG motif of the Arabidopsis LBD proteins (Shuai et al., 2002), the proline (P) and the glycine (G) were completely conserved in all predicted grapevine proteins. The conserved proline residue in the GAS block was demonstrated in Arabidopsis to be essential in the biological function of the LBD proteins since their replacement by leucine residues precludes LBD18-dependent control of the lateral root development via inhibition of the DNA-binding activity (Lee et al., 2013b). Valine (V) and leucine residues in the GAS block, as well as a glutamine (Q) in the leucine zipper-like motif, were found to be needed for motor organ specification in pea (Chen et al., 2012). As observed for other plant species, leucine zipper-like motif (LX6LX3LX6L) was observed only in Class I VviLBD proteins and absent in Class II proteins, which suggests distinct functions of both classes. N and C terminals beyond the 3 blocks were not conserved at all among any sequences indicating that they probably play only a marginal role in protein function (Supplementary Figure S3.2). It is, however, noteworthy that none of class II proteins presents an N-terminal, but this is not specific of the class; other class I proteins do not have it either. Class If protein present a longer, serine-enriched N terminal.

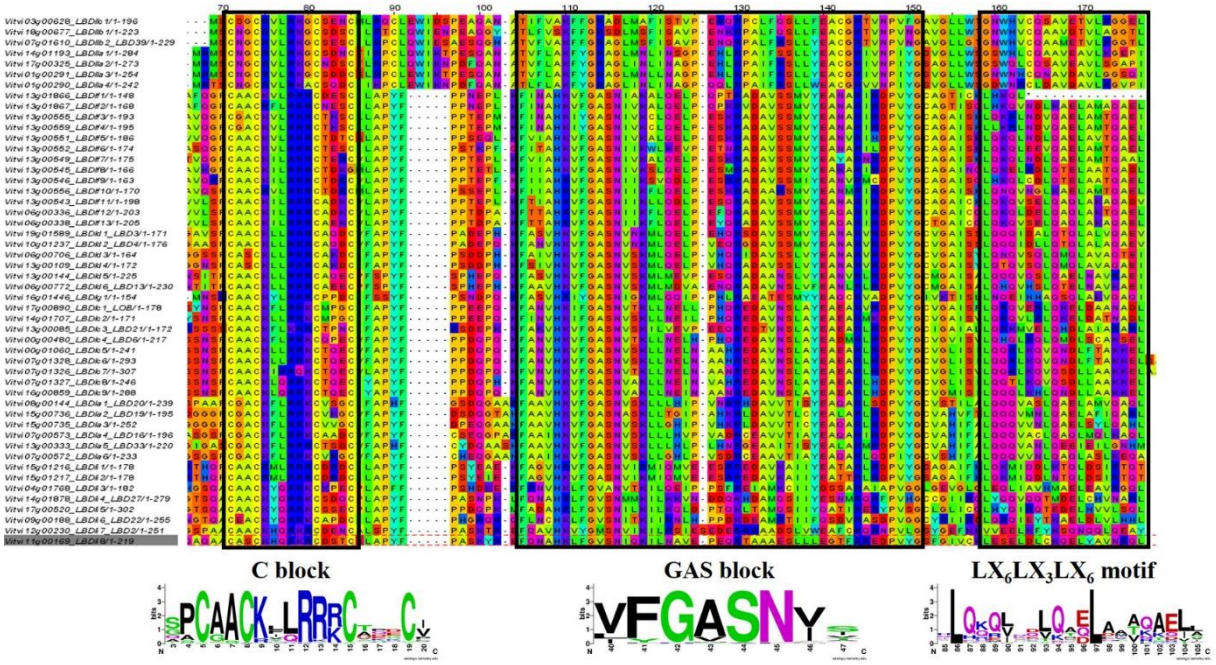

Figure 3.2. VviLBD protein alignment and motif analysis. Conserved domains were highlighted with black boxes. CX2CX6CX3C zinc finger-like domain was conserved in all 50 predicted VviLBD protein sequences while the leucine zipper-like motif (LX6LX3LX6L) was observed only in the class I VviLBD proteins. Details on protein structure are shown in Supplementary Figure S3.1.

The orthologous relationship of *LBD* genes in *V. vinifera* and other plant species was analyzed as previously described (Grimplet et al., 2016; Figure 3.3). Orthologous relationships were classified into two categories depending on whether or not a one-to-one relationship with a given species gene was detected. Since the 3 blocks previously mentioned were highly conserved, homology between a grapevine gene and many LBD genes was systematically detected, except for *VviLBDIi7* with most monocot species (in black in Figure 3.3). Twenty genes showed a one-to-one orthologue relationship with an Arabidopsis gene when the comparison was carried out only with Arabidopsis. These genes likely correspond to well-conserved functions between both species.

In this context, a phylogenetic tree considering several mono and dicotyledonous species was constructed to identify genes with widely conserved functions among species (Figure 3.3, Supplementary Figure S3.3). *VviLBDIa3, VviLBDIc1, VviLBDIc4, VviLBDId5, VviLBDIIa1,* and *VviLBDIIb2* presented orthologues at least in 88% of the species selected for comparison and could be involved in evolutionarily conserved functions.

This analysis did not detect orthologs for seventeen *LBD* genes, while less than five orthologs were detected for six genes, mainly belonging to subclass If and Class II. These results may indicate that those proteins play a specific role in grapevine, and in fact, Class IIc seems to be a *Vitis vinifera* species-specific subgroup. Regarding *VviLBDIa2, VviLBDIa6* and *VviLBDc3,* they might have evolved after the monocot-dicot divergence since no orthologs were identified for them in the analyzed monocot species. Supplementary Figure S3.3 shows a cluster of the *Vitis* genes from the LBD1f subclass indicating a possible duplication event that appeared later and might be specific of the *Vitis* genus. Additionally, a Ka/Ks analysis was performed using the Ka/Ks calculation tool (http://services.cbu.uib.no/tools/kaks) on all the orthologs detected in the species for each grapevine gene, but no positive selection involving a grapevine gene in our gene set could be detected (no branch showed Ka/Ks$\gg$1, Supplementary Table S3.1).

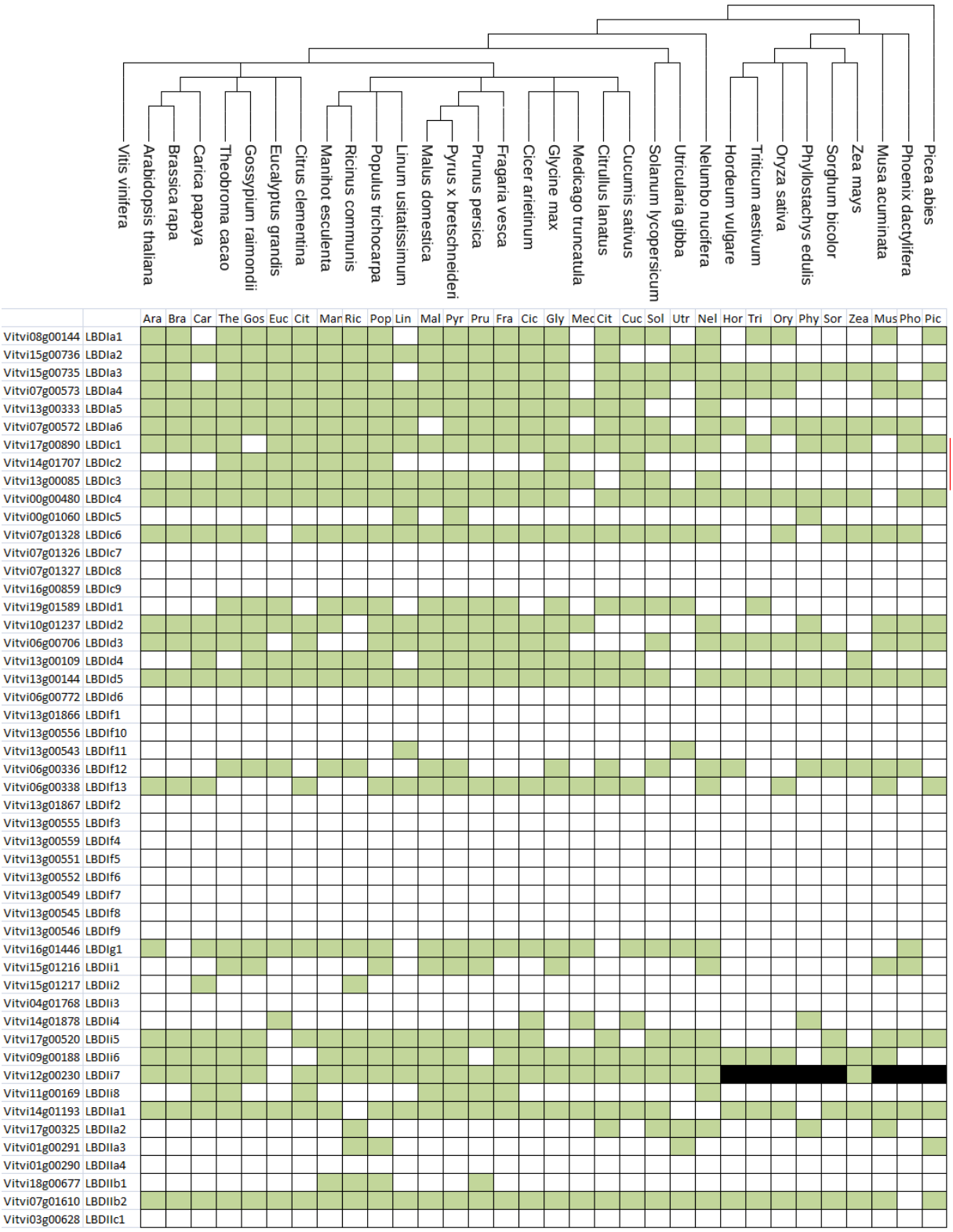

Figure 3.3. Grapevine *LBD* genes orthology against plant species with sequenced genomes. Green color represents one-to-one orthologs in the species (ortholog one-to-one = best match in the species that has the grapevine deduced protein as the best match in grapevine); white color represents no one-to-one homology match, and black color represents no match in the species.

3.3.3. Chromosomal location of the LBD genes.

Grapevine *LBD* genes are unevenly distributed among the nineteen chromosomes. They are located in almost all chromosomes, except on chromosomes 2, 5, and 11 (Figure 3.4). Two genes, *LBDIc4 LBD6* and *LBDIc5*, were located on two scaffolds not assembled yet into any chromosome (they appear in the fictional chromosome "Unknown"). The highest number of *VviLBD* genes (15) was located on chromosome 13. The high number of *LBD* genes in this chromosome is mainly due to tandem repetition of genes belonging to the same subclass, in particular, subclass *LBD* If genes. As highlighted by the orthology analysis, this duplication of class f probably occurred recently in *Vitis* since no ortholog was found in any other species. In contrast, chromosomes 3, 4, 8, 9, 10, 11, 12, 18, and 19 all carried a single *LBD* gene.

LBD genes belonging to the same subclass were located in chromosomal regions that were previously identified as paralogous segments resulting from ancestral polyploidization events (Jaillon et al., 2007; Velasco et al., 2007). In this way, *LBD* genes from subclass If are located in chromosomes 13 and 6, though LBD1f in chr13 was located mostly just beside the presumed paralogous segment (Figure 3.4). This is highly similar to what was obtained in a previous study for the GRAS sub-family LISCL (Grimplet et al., 2016). The LISCL genes are also duplicated in the same area close to the paralogous region and have paralogs in chr06. LBD1f and LISCL are at the same distance in chr13 and chr06 (1.7 Mb). It is possible that this area belongs actually to the paralogous region since the paralog analysis was performed in the very original 8X version of the genome (Jaillon et al., 2007) and might need an update. Class II genes are specific of two groups of paralogous segments, one group on chromosomes 1, 14, and 17 and another group on chromosomes 3, 7, and 18. This indicates that all these subclasses predate the ancestral polyploidization events and likely play specific roles in grapevine since their functions were not redundant and were not discarded throughout evolution.

In addition, there are also tandem repetitions of genes belonging to different subclasses, like *VviLBDIa5, VviLBDId4-5,* and *VviLBDIc3*. These data revealed that segmental duplication and tandem duplications contributed significantly to the expansion and evolution of the *LBD* gene family

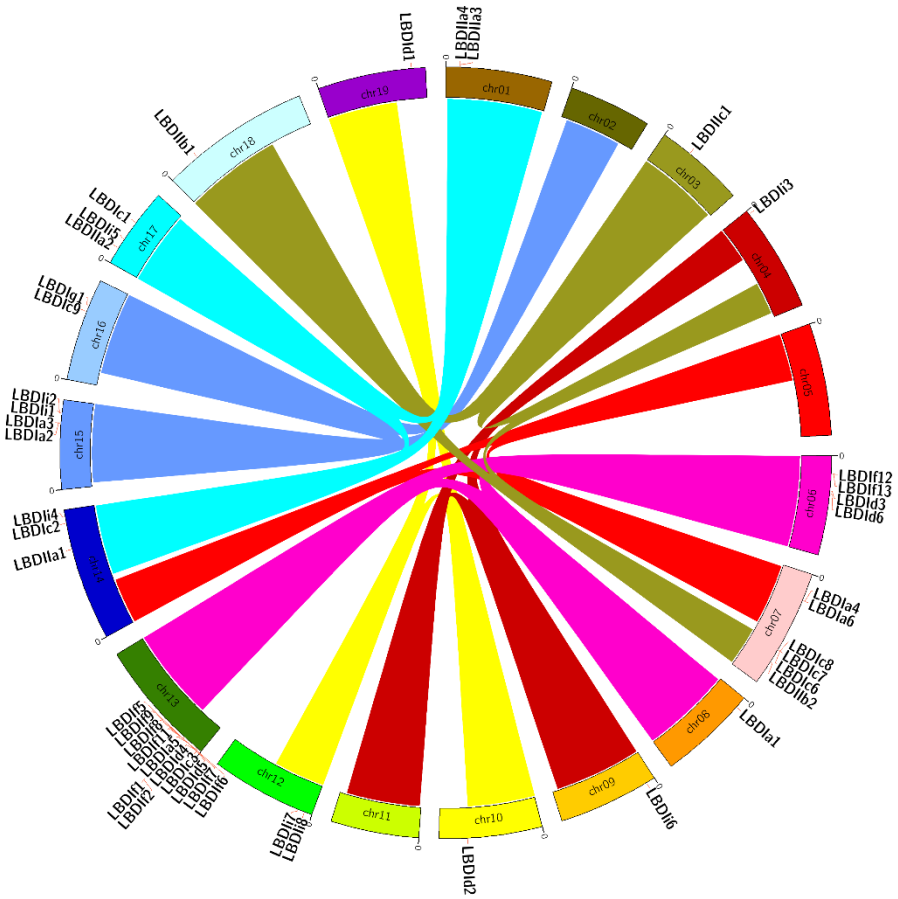

Figure 3.4. Chromosomal localization of grapevine *LBD* genes. Links with the same colors in different chromosomes show previously described paralogous regions (Lee et al., 2009). *LBD* genes from the same subclass were located in chromosomal regions that were previously identified as paralogous segments.

3.3.4. *Cis*-acting regulatory elements in promoter regions.

Analysis of *cis*-regulatory elements in the *VviLBD* promoter regions was performed using the PlantCARE (Supplementary Figure S3.4; Supplementary Table S3.2) and PlantPAN databases (Supplementary Figure S3.5; Supplementary Table S3.3). In addition to the core *cis*-elements, including the TATA box and CAAT box motifs presented in all promoter regions (data not shown), several regulatory motifs were identified and are associated with light regulation (BOX I, BOX 4, ACE, MRE), low temperature and heat stress responses (LTR, HSE), defense and stress responses (e.g., TC-rich repeats), hormonal regulation such as salicylic acid (e.g., TCA-element, CA-element), methyl jasmonate (e.g., CGTCA-motif), ethylene (e.g., ERE), auxin (AuxRR-core, TGA-element), abscisic acid (ABRE, motif IIb, CE3), gibberellin (P-box, TATC-box, GARE-motif) and regulatory motifs related to tissue-specific expression (e.g., Skn-1_motif, motif I, as1, GCN4_motif, RY-element) or developmental processes/ cell differentiation (HD-Zip 1, HD-Zip 2). Several transcription

factor binding sites were also identified, which have been widely associated with developmental processes and with biotic and abiotic stress responses, namely AP2/ERF, NAC, C2H2, SBP, WRKY, Myb, bZIP, and bHLH binding sites. Furthermore, these *cis*-regulatory elements were enriched in *LBD* promoter regions (Figure 3.5; at P-value < 0. 01). Interestingly, analyses of *de novo* motifs using the Homer platform enabled the identification of a new motif GGTTGAATACAC as being enriched in *VviLBD* promoters (Supplementary Table S3.4). A similar known motif to this one was identified as GATGGAATAC (Supplementary Table S3.4). It should be highlighted that the enrichment in MYB binding sites is known to regulate the expression of genes involved in phenylpropanoid metabolism. Interestingly, *VviLBDIf6* co-expressed with a gene coding for MYB divaricate and *VviLBDIa1* with a gene coding for a bHLH family transcription factor (Table 3.2). The bHLH048 transcription factor was already found to interact with the AtASL4 from Arabidopsis, and its interaction reduces the affinity of *LOB* gene for its 6-bp GCGGCG consensus DNA motif (Husbands et al., 2007).

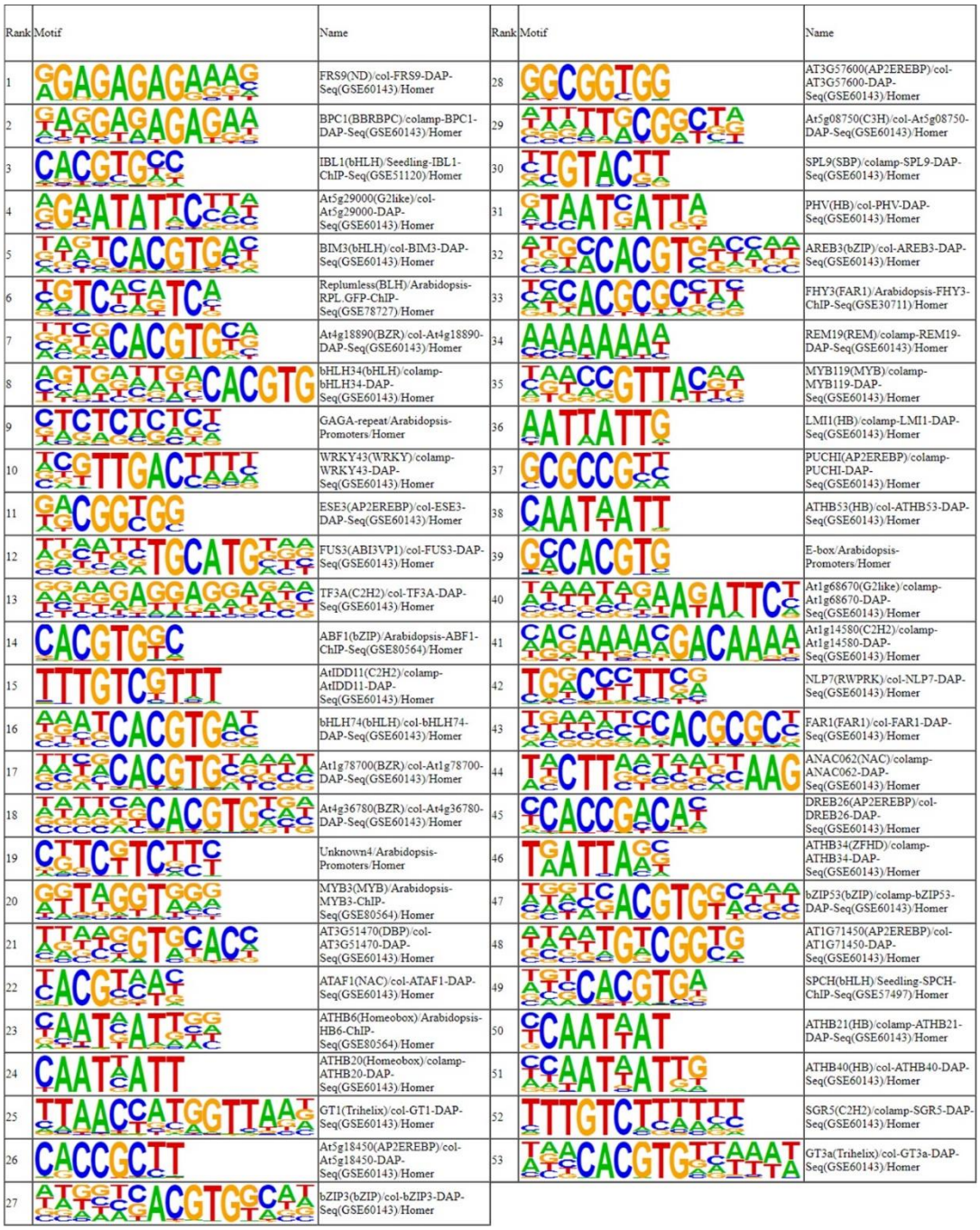

Figure 3.5. Enrichment of motifs on promoter regions of grapevine *LBD* genes. Several transcription factor binding sites were identifed as enriched: FRS9 (ND); BPC1 (BBRBPC); IBL1 (bHLH); At5g29000 (G2like); BIM3 (bHLH); Replumless (BLH); At4g18890 (BZR); bHLH34 (bHLH); GAGA-repeat; WRKY43 (WRKY); ESE3 (AP2EREBP); FUS3 (ABI3VP1); TF3A (C2H2); ABF1 (bZIP); AtIDD11 (C2H2); bHLH74 (bHLH); At1g78700 (BZR); At4g36780 (BZR); Unknown4; MYB3 (MYB); AT3G51470 (DBP); ATAF1 (NAC); ATHB6 (Homeobox); ATHB20 (Homeobox); GT1 (Trihelix); At5g18450 (AP2EREBP); bZIP3 (bZIP); AT3G57600 (AP2EREBP); At5g08750 (C3H); SPL9 (SBP); PHV (HB); AREB3 (bZIP); FHY3 (FAR1); REM19 (REM); MYB119 (MYB); LMI1 (HB); PUCHI (AP2EREBP); ATHB53 (HB); E-box; At1g68670 (G2like); At1g14580 (C2H2); NLP7 (RWPRK); FAR1 (FAR1); ANAC062 (NAC); DREB26 (AP2EREBP); ATHB34 (ZFHD); bZIP53 (bZIP); AT1G71450 (AP2EREBP); SPCH (bHLH); ATHB21 (HB); ATHB40 (HB); SGR5 (C2H2); GT3a (Trihelix).

3.3.5. Expression analysis of grapevine *LBD* genes.

Tree distinct approaches were performed to characterize *LBD* genes expression in grapevine: (i) an atlas of expression of the *LBD* genes was constructed based on the absolute value of gene expression published in the grapevine gene expression atlas (Fasoli et al., 2012) (Figure 3.6). Plant Ontology (PO) was attributed when a gene was clearly expressed in a given tissue. (ii) co-expression analysis was performed based on the same original data using relative values of expression of all genes, centered on the average expression (Supplementary Table S3.5). The main objectives of this analysis were to determine expression patterns and to identify genes that were following the same pattern as the *LBD* genes and that could be under the same regulatory elements, or under the regulation of the *LBD* gene itself. The results presented in Table 3.2 revealed that twelve genes showed a correlation with other genes with a Pearson Correlation Coefficient (PCC) threshold of 0.2. Finding the optimal PCC threshold to retrieve functionally related genes was affected by the method of gene expression database construction and the target gene function (Obayashi and Kinoshita, 2009), but the PCC that was chosen was very stringent. (iii) public expression data was analyzed in order to identify the behavior of *LBD* genes during berry development and ripening and upon abiotic and biotic stress conditions (Figure 3.7). Figure 3.7 presented the expression value among the experiments where the difference in expression of *LBD* genes was detected.

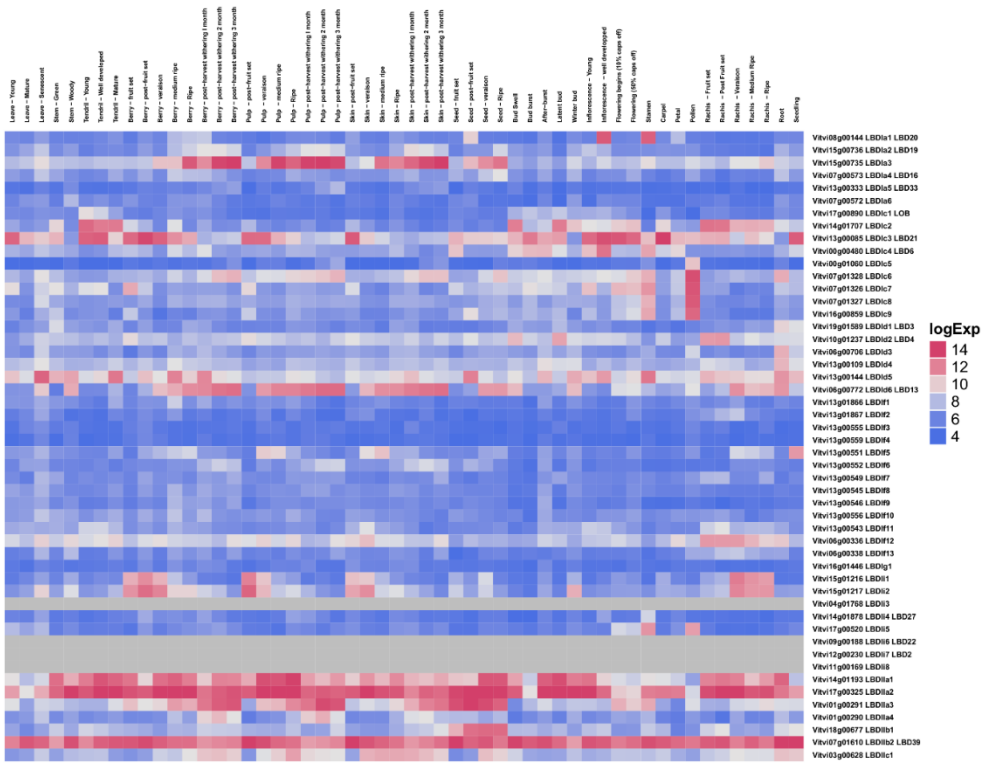

Figure 3.6. Expression of *LBD* genes in grapevine tissues. Gradient color is expressed in RMA-normalized intensity value on the Nimblegen microarray.

Table 3.2. Co-expression analysis of the *VviLBD* genes. For some genes, the list of co-expression is not complete. Further details are presented in Supplementary Table 3.2.

Unique_ID/Nimblegen probeset	Functional annotation	Functional Categories
VIT_15s0046g00230	**VviLBDIi1**	**LOB domain family transcription factor**
VIT_15s0046g00240	**VviLBDIi2**	**LOB domain family transcription factor**
VIT_01s0011g03540	**VviLBDIIa3**	**LOB domain family transcription factor**
VIT_10s0003g03490	GA 2-oxidase	Metabolism. Secondary metabolism. Terpenoid metabolism. Diterpenoid metabolism. Diterpenoid biosynthesis
VIT_14s0006g02950	**VviLBDIIa1**	**LOB domain family transcription factor**
VIT_12s0057g00170	Wound-induced	Response to stimulus. Stress response. Abiotic stress response. Wounding
VIT_07s0197g00040	**VviLBDIc7**	**LOB domain family transcription factor**
VIT_07s0031g02270	Tonoplast monosaccharide transporter2	Transport overview. Electrochemical Potential-driven Transporters. Porters. Major Facilitator Superfamily. Sugar Porter
VIT_14s0066g00680	**VviLBDIc2**	**LOB domain family transcription factor**
VIT_11s0016g05450	Equilibrative nucleoside transporter ENT8 splice variant	Transport overview. Electrochemical Potential-driven Transporters. Porters. Equilibrative Nucleoside Transporter
VIT_01s0011g03530	**VviLBDIIa4**	**LOB domain family transcription factor**
VIT_08s0007g04480	Pectinesterase family	Cellular process.Cellular component organization and biogenesis.Cell wall organization and biogenesis.Cell wall metabolism.Cell wall modifcation.Pectin modification
VIT_06s0004g07790	**VviLBDId6 LBD13**	**LOB domain family transcription factor**
VIT_02s0025g02940	Cafeic acid O-3-methyltransferase	Metabolism. Secondary metabolism. Phenylpropanoid metabolism. Phenylpropanoid biosynthesis
VIT_12s0028g03580	Lectin-receptor like protein kinase 3	Signaling. Signaling pathway. Protein kinase
VIT_14s0068g01360	GEM-like protein 5	Cellular process. Cell growth and death
VIT_02s0025g02920	Quercetin 3-O-methyltransferase 1	Metabolism. Secondary metabolism. Phenylpropanoid metabolism. Flavonoid metabolism. Flavonoid biosynthesis
VIT_15s0048g00830	**VviLBDIa3**	**LOB domain family transcription factor**
VIT_18s0001g15390	Gaiacol peroxidase	Metabolism. Primary metabolism. Amino acid metabolism. Aromatic amino acid metabolism. Phenylalanine metabolism. Phenylalanine biosynthesis
VIT_17s0000g09030	Disease resistance protein (NBS-LRR class)	Diverse functions. Gene family with diverse functions. NBS-LRR superfamily
VIT_15s0048g00500	Pectinesterase family	Cellular process. Cellular component organization and biogenesis. Cell wall organization and biogenesis. Cell wall metabolism. Cell wall modification. Pectin modification
VIT_13s0019g03780	**VviLBDIf6**	**LOB domain family transcription factor**
VIT_07s0031g02280	MYB divaricata	Development. Reproductive development. Flower development
VIT_08s0056g01650	**VviLBDIa1 LBD20**	**LOB domain family transcription factor**
VIT_11s0103g00200	Anthranilate N-benzoyltransferase	Metabolism. Primary metabolism. Amino acid metabolism. Aromatic amino acid metabolism. Aromatic amino acid biosynthesis
VIT_01s0127g00860	Aborted microspores AMS	Regulation overview. Regulation of gene expression. Regulation of transcription. Transcription factor. bHLH family transcription factor
VIT_18s0001g15690	Endo-1,4-beta-glucanase	Cellular process. Cellular component organization and biogenesis. Cell wall organization and biogenesis. Cell wall metabolism. Cell wall catabolism. Cellulose catabolism
VIT_18s0001g15680	Cellulase	Cellular process. Cellular component organization and biogenesis. Cell wall organization and biogenesis. Cell wall metabolism. Cell wall catabolism. Cellulose catabolism
VIT_15s0021g02170	Chalcone and stilbene synthase	Metabolism. Secondary metabolism. Phenylpropanoid metabolism. Flavonoid metabolism. Flavonoid biosynthesis
VIT_17s0000g05490	**VviLBDIi5**	**LOB domain family transcription factor**
VIT_09s0002g04380	Plastidic glucose transporter 2	Transport overview. Electrochemical Potential-driven Transporters. Porters. Major Facilitator Superfamily. Sugar Porter
VIT_12s0059g02500	Constans-like 11	Development. Reproductive development. Flower development

VIT_18s0001g13580	Kinesin motor protein	Cellular process. Cellular component organization and biogenesis. Cytoskeleton organization and biogenesis. Microtubule organization and biogenesis. Microtubule-driven movement
VIT_03s0063g00510	Leucine-rich repeat	Diverse functions. Gene family with diverse functions. NBS-LRR superfamily
VIT_06s0009g01830	Invertase, neutral/alkaline	Metabolism. Primary metabolism. Carbohydrate metabolism. Monosaccharide metabolism. Galactose metabolism
VIT_07s0031g01870	Zinc finger (CCCH-type) family protein	Regulation overview. Regulation of gene expression. Regulation of transcription. Transcription factor. C3H family transcription factor
VIT_00s2422g00010	Hexokinase-2	Metabolism. Primary metabolism. Carbohydrate metabolism. Glycolysis Gluconeogenesis
VIT_00s0288g00050	V-type H G +-transporting ATPase subunit	Metabolism. Primary metabolism. Generation of metabolite precursors and energy. Electron transport. Respiratory-chain phosphorylation
VIT_19s0014g01240	Morphogenesis of root hair 1 MRH1	Development. Root development
VIT_18s0122g00910	Mlo5	Cellular process. Cell growth and death. Cell death
VIT_17s0000g07750	Zinc fnger protein 5	Regulation overview. Regulation of gene expression. Regulation of transcription. Transcription factor. C2H2 family transcription factor
VIT_07s0005g01640	Feronia receptor-like kinase	Signaling. Signaling pathway. Protein kinase
VIT_00s0225g00170	Peroxidase	Metabolism. Primary metabolism. Amino acid metabolism. Aromatic amino acid metabolism. Phenylalanine metabolism. Phenylalanine biosynthesis

3.3.5.1. Tissue-specific gene expression.

Based on the *V. vinifera* cv. Corvina gene expression atlas (Fasoli et al., 2012), several *LBD* genes showed a strong tissue specificity of expression, with the majority of Class I genes being poorly expressed in the different tissues (Figure 3.6). *VviLBDIa3* and *VviLBDId6* were highly expressed mainly in ripe berry tissues. *VviLBDId6* was shown to be co-expressed (Table 3.2, Supplementary Table S3.5) with genes involved in phenylpropanoid metabolism, including two caffeic acid O-3-methyltransferase genes (VIT_02s0025g02940 and VIT_02s0025g02930) and a quercetin 3-O-methyltransferase gene (VIT_02s0025g02920), as well as with signaling and cell growth and death-related genes (VIT_12s0028g03580 and VIT_14s0068g01360, respectively). *VviLBDIc3* had high expression in young leaves, young and well-developed tendril, inflorescences, and in berry tissues mainly at the beginning of fruit development (green and *véraison* stages). In addition, transcripts corresponding to subclass Ic genes, *VviLBDIc6-9*, seemed to be more abundant in pollen and stamen.

Interestingly, *VviLBDIa1* was only expressed in well-developed inflorescence and stamen and may have a specific function in the development of these tissues; it also co-expressed with genes involved in the cell wall and secondary metabolism and transport (VIT_18s0001g15690, VIT_18s0001g15680, VIT_11s0103g00200, VIT_19s0015g00960). *VviLBDIc7* was co-expressed with *TONOPLAST MONOSACCHARIDE TRANSPORTER 2* (VIT_07s0031g02270). The class II genes *VviLBDIIa1, VviLBDIIa2*, and *VviLBDIIb2* were abundantly expressed in almost all grapevine tissues, and, *VviLBDIIa3* was more abundant in

seeds and post-harvest berry tissues. Differential expression of *LBD* genes in diverse tissues were also observed for other plant species including Arabidopsis, rice, and maize (Shuai et al., 2002; Yang et al., 2006; Zhang et al., 2014).

3.3.5.2. Gene expression during berry development and ripening.

Expression studies regarding berry development and ripening revealed the involvement of *LBD* genes in different stages (Figure 3.7). *VviLBDIc1* gene was highly expressed after EL31 stage (Coombe, 1995), i.e., pea-size berries, both in cv. Cabernet Sauvignon and cv. Chardonnay (Figure 3.7). However, this expression profile can be cultivar and/or season dependent. In cv. Trincadeira, *VviLBDId6* was expressed along berry ripening, as previously mentioned for cv. Corvina (Figure 3.7). The same tendency was observed in cv. Carignan, in which *VviLDBId6* expression was up-regulated at the *véraison* stage (Table 3.3). The same holds true for *VviLBDIa3*, which showed up-regulation after the onset of ripening in cv. Trincadeira (Figure 3.7) and was co-expressed with genes involved in stress response (Table 3.2, Supplementary Table S3.5), namely a Disease Resistance protein (NBS-LRR class) (VIT_17s0000g09030) and a Gaiacol peroxidase (VIT_18s0001g15390). On the other hand, *VviLBDIi1* was down-regulated in advanced ripening stages. In cv. Carignan, a down-regulation was observed at *véraison* stage (Table 3.3).

Concerning class II genes, *VviLBDIIa1, VviLBDIIa2, VviLBDIIa3* seem to play a role in grape ripening. Furthermore, *VviLBDIIa1* co-expressed with wound-induced genes involved in abiotic stress response and *VviLBDIIa3* co-expressed with a gene coding for GA 2-oxidase (VIT_10s0003g03490). Moreover, *LBDIf5* and *LBDIIc1* were shown to be up-regulated at the *véraison* stage only in cv. Carignan, suggesting a cultivar specific response (Table 3.3). On the opposite side, *LBDIc3* and *LBDIc7* were shown to be down-regulated upon *véraison* (Figure 3.7, Table 3.3).

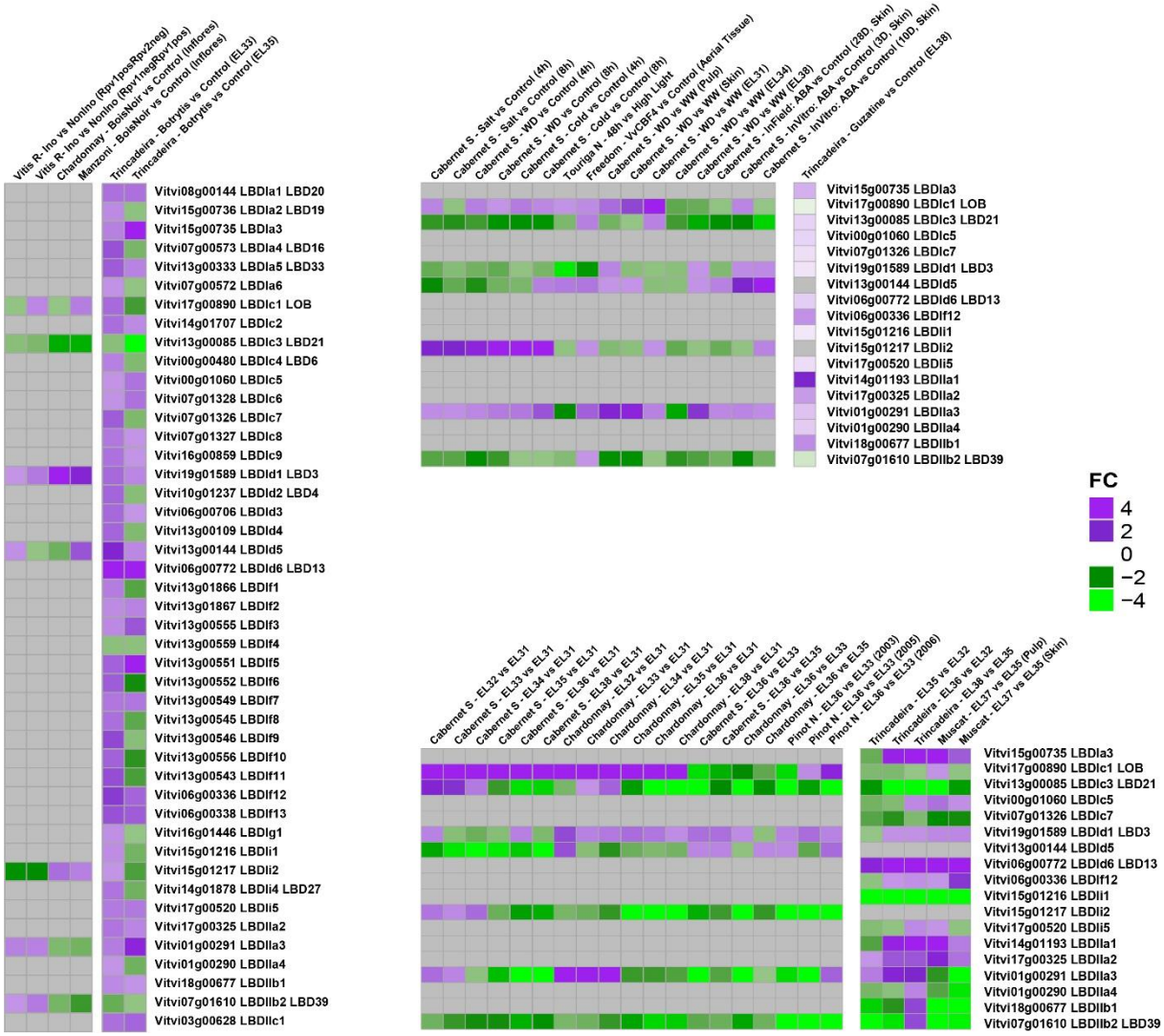

Figure 3.7. Expression of *LBD* genes during grape berry development and ripening, and upon abiotic and biotic stresses. Left experiments of each heatmap were performed with GeneChip microarrays, and the right experiments were performed with GrapeGen microarray. Grape berry development: developmental stages from EL31 to EL38; cultivars Cabernet Sauvignon, Chardonnay, Pinot Noir, Trincadeira, and Muscat. Abiotic stress experiments: salt, cold, water deficit, high light, ABA. Biotic stress experiments: *P. viticola*, *BoisNoir*, and *Botrytis cinerea*.

3.3.5.3. Gene expression upon abiotic stress.

Expression analysis concerning abiotic stress conditions (Figure 3.7) revealed that *VviLBDIi2* was up-regulated under salt, cold, and water deficit conditions in shoot tips. *VviLBDId5* expression in berry skin showed a positive response to *in vitro* ABA treatment. *VviLBDIIa3* was also up-regulated in pulp and skin submitted to water deficit. Interestingly, *VviLBDIIa1* responded to guazatine treatment, an inhibitor of polyamine oxidase involved in polyamine catabolism (Agudelo-Romero et al., 2014).

On the other hand, some *VviLBD* genes presented mostly down-regulation upon abiotic stress, such as *VviLBDIc3* and *VviLBDIIb2. VviLBDId1* was strongly down-regulated after 48 h of high light exposure.

3.3.5.4. Gene expression upon biotic stress.

Regarding biotic stress conditions (Figure 3.7), *VviLBDIi2* was down-regulated in partially and completely resistant plants (resistance genes named Rpv1 and Rpv2) when inoculated with *Plasmopara viticola*. *VviLBDId1* was up-regulated in inflorescences presenting Bois noir disease, with higher expression in cv. Chardonnay. On the other hand, *VviLBDIc3* was down-regulated in the same conditions. This gene was also down-regulated in grape berries infected with *Botrytis cinerea*, with lower expression after long exposure (*véraison* stage). *VviLBDId6* and *VviLBDIIa1* were strongly up-regulated after *Botrytis* infection. *VviLBDIIa1* co-expressed with six wound-induced genes, as previously mentioned (Table 3.2, Supplementary Table S3.5). *VviLBDIa3, VviLBDIf5,* and *VviLBDIIa3* were up-regulated upon *Botrytis* infection with higher expression at *véraison* stage. Moreover, *LBDIIa3* was also over-expressed in response to *Erysiphe necator* infection at the green stage (Table 3.3). The majority of *VviLBDs* seemed to participate in the early response towards *Botrytis* attack. Upon *E. necator* infection, *LBDIa1*, *LBDIf13*, and *LBDIIb1* genes were up-regulated only at the green stage (EL33); however, *LBDId4* was overexpressed at the *véraison*, suggesting that *LBDId4* might be involved in later responses (Table 3.3).

Table 3.3. Differently expressed *LBD* genes in powdery mildew (caused by *Erysiphe necator*) infected and control *V. vinifera* cv. Carignan grape berries at green (EL33) and *véraison* (EL35) stages. Reported changes correspond to significant up- or down-regulation (FDR≤0.05 and fold change ≥ 2 or ≤ -2). Four comparisons were performed: EL33 PM infected versus EL33 control; EL35 PM infected versus EL35 control; EL35 control versus EL33 control; and, EL35 PM infected versus EL33 PM infected.

Gene ID V1	Locus ID	Short Name	EL33 (PM vs Control)	EL35 (PM vs Control)	Control (EL35 vs EL33)	PM Infected (EL35 vs EL33)
VIT_08s0056g01650	Vitvi08g00144	LBDIa1 LBD20	19.92			-15.81
VIT_13s0067g01450	Vitvi13g00085	LBDIc3 LBD21			-2.29	-3.23
VIT_07s0197g00040	Vitvi07g01326	LBDIc7			-3.02	-2.45
VIT_13s0067g01880	Vitvi13g00109	LBDId4		5.63		3.99
VIT_06s0004g07790	Vitvi06g00772	LBDId6 LBD13			6.31	16.13
VIT_06s0004g03350	Vitvi06g00338	LBDIf13	6.15			
VIT_13s0019g03760	Vitvi13g00551	LBDIf5			7.78	39.14
VIT_15s0046g00230	Vitvi15g01216	LBDIi1			-3.48	-3.10
VIT_01s0011g03540	Vitvi01g00291	LBDIIa3	5.20			-4.88
VIT_18s0001g09250	Vitvi18g00677	LBDIIb1	5.36			-9.11
VIT_07s0129g00330	Vitvi07g01610	LBDIIb2 LBD39			-2.24	
VIT_03s0091g00670	Vitvi03g00628	LBDIIc1			2.44	3.59

3.4. Discussion

Prediction of putative biological functions for a given gene family can be approached based on genomic and transcriptomic available data with improved bioinformatics tools. In this study, we performed an extensive analysis of the *LBD* genes on the 12x *Vitis vinifera* genome sequence based on the isolation of the complete set of genes identified in PN40024. Characterization of *LBD* gene family and their putative functions was performed in grapevine based on detailed gene structure and expression analyses, chromosome localization, and comparative phylogenetic analyses with other sequenced genomes from different monocot and eudicot species.

3.4.1. *LOB domain* gene family in grapevine.

The *LOB domain* gene family, also known as *ASYMMETRIC LEAVES2*-like (*ASL*) gene family, was firstly described in the past decade (Iwakawa et al., 2002; Shuai et al., 2002) and several studies have been made to unveil their role in plant processes. Specific LBD proteins characterized in Arabidopsis and in crop plants, including rice, seem to display a wide functional diversity. They were found involved in the regulation of several developmental processes, namely meristem programming, leaf patterning, inflorescence development, embryogenesis, lateral root formation, vascular patterning, as well as metabolic processes such as anthocyanin and nitrogen metabolisms (Majer and Hochholdinge, 2011; Xu et al., 2016). More recently, they have also been associated with biotic stress responses (Cabrera et al., 2014; Hu et al., 2014; Thatcher et al., 2012b, 2012a).

The exhaustive analysis of the grapevine *LBD* genes performed in this study has led to the identification of 50 *LBD* genes. The N-terminal LOB domain that characterizes this gene family was identified in all predicted proteins. The *VviLBD* genes were located to sixteen of the nineteen grapevine chromosomes. Phylogenetic analysis and evolutionary relationships divided the *LBD* gene family into two classes, Class I and Class II, as previously observed to other plant species, characterized by the presence or absence of functional leucine-zipper-like domains, respectively (Iwakawa et al., 2002; Shuai et al., 2002; Wang et al., 2013; Yang et al., 2016, 2016; Zhang et al., 2014). The majority of *VviLBD* genes belongs to Class I. Grapevine *LBD* genes were further clustered into nine subclasses, six in Class I and three in Class II. Minor clades of the two major classes were also identified in other plant species: class I was divided into five subgroups in rice and maize, four in Arabidopsis, *Lotus japonicus* and *Medicago truncatula*, and seven in *Malus domestica* (Matsumura et al., 2009; Schnable et al., 2009; Wang

et al., 2013; Zhang et al., 2014). Class II was divided into two subclasses in apple and maize (Wang et al., 2013; Zhang et al., 2014).

The gene structure analysis revealed that 74% (37 out of 50) of the *VviLBD* genes contained two exons, as the majority of *LBD* genes in other plant species (Matsumura et al., 2009; Wang et al., 2013; Yang et al., 2006), indicating a conserved structure during evolution. On the other hand, the highly variable C-terminal domain in LBD proteins from grapevine and other plant species indicate functional diversity associated with this gene family (Matsumura et al., 2009). Experiments of LOB domain swapping for the *AS2* gene revealed that, despite the high similarity, the domain cannot be functionally replaced by a LOB domain of other family members indicating that dissimilar amino acid residues in the N-terminal are also important for the functional specificities of these transcription factors family members (Matsumura et al., 2009). The highly conserved C-block (CX2CX6CX3C) domain is present in all grapevine LBD proteins reinforcing its functional importance mainly associated with the DNA-binding process (Matsumura et al., 2009; Shuai et al., 2002). Class I proteins presented the leucine-zipper-like motif, which includes five hydrophobic amino acids (valine, isoleucine, leucine) separated by six variable amino acid residues and has been linked to protein-protein interaction.

The grapevine *LBD* gene family with 50 members is larger than the 43 *LBD* genes of Arabidopsis, the 24 in barley, the 31 in mulberry; the 35 in rice, 36 in *Sorghum bicolor*; the 38 in *Lotus japonicus*, and the 44 in maize (Guo et al., 2016; Iwakawa et al., 2002; Luo et al., 2006, 2016; Shuai et al., 2002; Wang et al., 2010; Yang et al., 2016, 2006; Zhang et al., 2014). *Medicago truncatula* and apple present a higher number of *LBD* genes, containing 57 and 58, respectively (Wang et al., 2013; Yang et al., 2016). With approximately the same genome size, *Vitis vinifera* harbors more *LBD* genes than *Lotus japonicus* (approximately 487 and 470 Mb, respectively). Despite *Malus domestica* had almost the double genome size of *Vitis vinifera*, this fruit tree species only contains eight more genes (Wang et al., 2013). Therefore, a large LBD density variation is observed among plant species.

Expansion of the grapevine *LBD* gene family likely took place by segmental/chromosomal duplication, as observed for other species from different taxonomic groups (Luo et al., 2016; Matsumura et al., 2009; Wang et al., 2013). Duplicated genes like *VviLBDIi1* and *VviLBDIi2* might show functional redundancy, as suggested by their similar expression profile or co-expression in several grapevine tissues. Their functional study might unveil the evolutionary role of gene duplication and their contribution in plant processes. Duplication events are more likely to be retained for gene families involved in signal transduction and transcriptional regulation (Blanc and Wolfe, 2004). Nevertheless, no

additional grapevine *LBD* genes co-expressed together which might indicate different functions or specialization in most cases. In fact, other likely duplicated genes such as *VviLBDIf12* and *VviLBDIf13*, showed a clear expression divergence in the expression analysis which might suggest their functional diversification. Tandem duplicated events were mainly associated with subclass If, which contained tandem repeated genes with high similarity. *VviLBDIf12* and *VviLBDIf13* presented several orthologs in other plant species, which might indicate conserved function. Nevertheless, for the remaining members of subclass If no ortholog was identifed in the studied species, suggesting grapevine-specific functions. Moreover, some genes from specific subclasses were found in paralogous regions of the grapevine genome derived from polyploidization event (Jaillon et al., 2007). Among them, subclass If had members in chromosomes 6 and 13, subclass IIa in chromosomes 1 and 17, and subclass IIb in chromosomes 7 and 18.

3.4.2. Expression patterns across a variety of tissues indicate roles of LBD genes in regulation of metabolism and organ differentiation.

VviLBD genes showed different expression patterns across the grapevine tissues. No subclass-specific expression pattern was observed, as occurred in other species, namely *L. japonicus*, *M. truncatula*, and apple, suggesting gene-specific function or localization regardless of the phylogenetic subclass (Wang et al., 2013; Yang et al., 2016). For example, *VviLBDIIb2* is highly expressed in almost all grapevine tissues whereas *VviLBDIIb1* seemed to be expressed only in seeds. *LBD38*, the Arabidopsis ortholog of *VviLBDIIb2*, is involved in nitrogen and anthocyanin metabolism, as well as their close homologs *LBD37* and *LBD39* (Rubin et al., 2009). Moreover, the *LBD37* rice ortholog, *OsLBD37/ASL39*, was also associated with nitrogen metabolism, particularly in nitrogen remobilization and senescence (Albinsky et al., 2010). This could suggest a conserved function of these genes across plant species and therefore, *VviLBDIIb2* could regulate nitrogen and anthocyanin metabolism in a wide range of grapevines tissues. In fact, orthologs for this gene were found in 32 out of the 33 plants studied (Figure 3.3).

Specific tissue expression patterns suggest the involvement of *VviLBDIa1* and *VviLBDIc3* in the development of floral organs. Interestingly, *VviLBDIa1* co-expressed with cell wall-related genes (endo-1,4-beta-glucanase, cellulose), secondary metabolism genes (anthranilate N-benzoyltransferase, chalcone, and stilbene synthase) and with a bHLH family transcription factor (*aborted microspores AMS*). In rice, down-regulation of an *LBD* gene (*OsIG1*) led to developmental abnormalities of various floral organs (Zhang et al., 2015), providing links

between LBD proteins and floral organ development as it could be the case in grapevine. Furthermore, in *Arabidopsis thaliana ASYMMETRIC LEAVES1* and *ASYMMETRIC LEAVES1 2* (*AS1* and *AS2*) and *JAGGED* (*JAG*) genes were shown to function in sepal and petal primordia to repress boundary-specifying genes for normal development of the organ (Xu et al., 2008). In grapevine, the ortholog of the Arabidopsis *LBD6/AS2* is *VvLBDIc4*, which is also expressed in flower organs but at a lower extent than *VviLBDIa1* and *VviLBDIc3*. Also noteworthy is the pollen-specific expression of *VviLBDIc6*, *VviLBDIc7*, *VviLBDIc8* and *VviLBDIc9*, which may have redundant functions. *VviLBDIc6* has orthologs in several species including *AtLBD36* which was previously shown to be expressed in pollen (Loraine et al., 2013).

3.4.3. LBD genes may be involved in berry development and ripening through interaction with growth regulators.

In grapevine, some *LBD* genes showed differential expression during fruit ripening, in particular, the up-regulated genes *VviLBDIa3*, *VviLBDId6*, and *VviBDIIa1*, and the down-regulated *VviLBDIc3*, *VviLBDIi1*, and *VviLBDIi2*. *VviLBDIIa3* was up-regulated at the initial development stages in cv. Chardonnay, suggesting an involvement in fruit set and early developmental stages characterized by intense cell division. Nevertheless, *VviLBDIIa3* showed higher expression on the ripe stage of cv. Trincadeira, suggesting an expression pattern dependent on the variety. This gene is also co-expressed with a gene coding for gibberellin 2-oxidase (VIT_10s0003g03490) that inactivates endogenous bioactive gibberellins (GAs), suggesting an involvement of *VviLBDIIa3* in GA metabolism during fruit-set. In cv. Corvina, *VviLBDIIa3* was highly expressed in seed at fruit set and post-fruit set stages but also in ripe and post-harvest berries (Figure 3.7). Little is known about the direct involvement of GAs on berry ripening; nevertheless, some evidence suggests a possible role in flowering and initial stages of berry development (Fortes et al., 2015). Additionally, differential accumulation of bioactive GAs was observed from flowering to fruit set, and this accumulation is finely regulated by the abundance and localization of GA oxidase transcripts (Giacomelli et al., 2013). Interestingly, *AtLBD40*, a close homolog of grapevine LBD subclass IIa genes, was reported to be a direct target of DELLA (growth-repressing transcription factor) in GA signaling pathway and to be down-regulated by gibberellin (Zentella et al., 2007).

VviLBDId6 was expressed in ripe and post-harvest berry tissues, and, also showed differential expression under biotic conditions. This gene was co-expressed with several genes involved in secondary metabolism (caffeic acid *O*-3-methyltransferase and quercetin 3-*O*-methyltransferase gene), signaling pathways (lectin-receptor like protein kinase 3) and cell

growth and death (GEM-like protein 5). The expression of the close Arabidopsis homolog, *AtLBD15/ALS11* leads to down-regulation of several cellulose synthase genes and is activated by a key regulator of secondary cell wall synthesis (Zhu et al., 2014).

In *Vitis vinifera, LBD1d6* and *LBDIa3* were identified as positive molecular markers of ripening stage in three Portuguese cultivars (Agudelo-Romero et al., 2013). Analysis of *cis*-acting elements suggests modulation of these genes by several growth regulators (ABA, methyl jasmonate, auxin, ethylene) and in response to stress (Supplementary Table S2). *VviLBDIa3* promoter showed a MYB binding site involved in flavonoid biosynthetic genes regulation. Interestingly, these two genes (VIT_06s0004g07790, VIT_15s0048g00830) were also identified as switch genes together with MYB transcription factors, cellulase, expansin B and caffeic acid 3-*O*-methyltransferase due to the fact that they are expressed at low levels in vegetative/green tissues and show a significant increase in mature/woody organs, suggesting a potential regulatory role during this developmental transition (Palumbo et al., 2014). The putative participation of *LBD* genes in fruit ripening, as suggested here, is additionally supported by studies in banana, where *MaLBD1/2/3* was found to be ripening inducible (Ba et al., 2014).

Interestingly, the promoter of *VviLBDIa3* was one of the promoters of LBD genes with AuxRR-core motif involved in auxin responsiveness. The Arabidopsis ortholog of *VviLBDIa3*, *LBD18/ASL20*, together with *LBD16* and *LBD*29, are key regulators of lateral root initiation/formation as direct targets of AUXIN RESPONSE FACTORs (Feng et al., 2012; Lee et al., 2009; Okushima et al., 2007). Arabidopsis *LBD16*, *17*, *18*, and *29* were also found to have an important role in *in vitro* callus formation induced by auxin (Fan et al., 2012). Furthermore, both Arabidopsis and banana *LBD* genes were shown to directly regulate expression of *EXPANSIN* genes, encoding cell wall-loosening factors (Ba et al., 2014; Kim and Lee, 2013; Lee et al., 2013a; Lee and Kim, 2013) that are also modulated during grape ripening (Fortes et al., 2011).

Other LBD genes, such as *VviLBDIi1*, were less expressed during grape ripening. In fact, this gene, as well as *VviLBD1c3*, were identified as negative biomarkers of ripening stage in three Portuguese cultivars (Agudelo-Romero et al., 2013). Additionally, *VviLBDIi1* possesses a *cis*-acting element involved in ABA responsiveness, a growth regulator that increases during ripening (Fortes et al., 2015), suggesting that this *LBD* gene might be negatively regulated by ABA. Moreover, *VviLBDIc7* were also shown to be repressed upon *véraison* in Trincadeira, Muscat, and Carignan varieties. *VviLBDIc7* seems to be co-expressed with a *Tonoplast*

Monosaccharide Transporter (VIT_07s0031g02270 - *TMT3*), which was previously reported to be down-regulated during ripening as well (Massonnet et al., 2017).

Brassinosteroids are steroidal plant hormones that have been proposed as ripening promoters in non-climacteric fruits, in particular grape berries (Fortes et al., 2015). The Arabidopsis *LOB* gene negatively regulates the accumulation of brassinosteroids in organ boundaries (Bell et al., 2012). *VviLBDIc1* is an ortholog of *LOB* and seems to be down-regulated during grape ripening as suggested here and in previous studies (Agudelo-Romero et al., 2013), and possibly interacts with brassinosteroids. However, it should be noted that at the pea-size stage of berry development *VviLBDIc1* expression seems to be very low compared to the following ripening stages highlighting the importance of conducting detailed temporal studies of gene expression.

Altogether *VviLBD1d6, VviLBDIa3,* and *VviLBD1c3* are robust candidates to participate in the regulation of the onset of the grape ripening program.

3.4.4. Expression of LBD genes upon abiotic and biotic stresses.

In grapevine, some class I genes showed differential expression under abiotic stress conditions. Particularly, *VviLBDIi2* is up-regulated under salt, cold, and water deficit conditions, *VviLBDId5* is up-regulated after *in vitro* ABA treatment, and *VviLBDId1*, down-regulated after 48 h of high light exposure. Interestingly, *VviLBDIi2* presents in his promoter a MYB binding site involved in drought-inducibility, *VviLBDId5* a *cis*-acting element involved in the abscisic acid responsiveness and *VviLBDId1* many elements involved in light responsiveness, suggesting the involvement of LBDs in abiotic stress response.

Few studies focused on the role of *LBD* genes in abiotic stress. However, in *Medicago truncatula*, *LBD1* gene was reported to have an important role in root architecture under salt stress (Ariel et al., 2010a). Additionally, MtHB1, an ABA and salinity responsive transcription factor, was found to directly recognize a specific *cis*-acting element in the *MtLBD1* promoter (Ariel et al., 2010b). By contrast, several *Sorghum bicolor LBD* genes were highly induced under salt and drought stress conditions, suggesting a role in abiotic stress response (Wang et al., 2010), whereas, in banana fruit, *MaLBD5* expression was induced by cold and methyl jasmonate treatment (Ba et al., 2016).

The majority of *VviLBDs* seem to participate in the early response towards *Botrytis* attack, as previously mentioned. However, *VviLBDId6* and *VviLBDIIa1* were strongly up-regulated after *Botrytis* infection both at EL33 (green berries) and EL35 (*véraison*), but not upon powdery mildew infection. *VviLBDIIa1* was also found co-expressed with six wound-induced genes, as

previously referred (Table 3.2, Supplementary Table S3.5). Interestingly, the promoter of this gene presents several *cis*-elements related to abiotic stress and methyl jasmonate responses. Jasmonates were previously proposed to be involved in grape response to *Botrytis* infection (Agudelo-Romero et al., 2015). Expression profiles of several Class II *AtLBD* genes revealed induction by pathogens, including necrotrophic fungal pathogens *Alternaria brassicicola*, and *B. cinerea*, root pathogen *Phytophthora parasitica* (oomycete) and the root-knot nematode *Meloidogyne incognita*, suggesting a role in plant defense mechanisms (Thatcher et al., 2012a). *VviLBDIIa3* seems to be involved in GA regulation of fruit set and fruit ripening but this gene was also up-regulated upon *Botrytis* infection, with higher expression at *véraison* stage, and in powdery mildew infected berries at green stage. Although gibberellins are mainly associated with plant growth and development, they have been recently related to response to pathogen attack (De Bruyne et al., 2014). Still, further studies are required to elucidate the role of GA in plant defense that remains very complex and unclear. Genes coding for gibberellin 20 oxidase were up-regulated after *B. cinerea* infection (Agudelo-Romero et al., 2015), suggesting activation of GA metabolism in defense response possibly with the involvement of *VviLBDIIa3*.

The involvement of the *LBD* genes in stress response has been poorly studied so far though some genes have been shown to play a role in disease susceptibility (Xu et al., 2016). *AtLBD20* was the first *LBD* gene associated with disease susceptibility (Thatcher et al., 2012b). In grapevine, *AtLBD20* homolog/ortholog did not show a relevant expression level in berries upon *B. cinerea* fungal infection; however, *VviLBDIa3*, belonging to the same clade, was up-regulated after long exposure to *B. cinerea* inoculation. In addition, *VviLBDIa3* gene was found to be co-expressed with several genes including an NBS-LRR gene (VIT_17s0000g09030) related to defense and a pectinesterase gene (VIT_15s0048g00500), involved in cell wall modification processes. Nucleotide-binding site (NBS) leucine-rich repeats (LRR) proteins are involved in the recognition of pathogen effectors with virulence functions (DeYoung and Innes, 2006).

Besides *VviLBDIa3* and *VviLBDIIa3, VviLBDIf5* was up-regulated upon *Botrytis* infection at *véraison* stage. Although no ortholog could be found for this gene, it belongs to the clade If that comprises the *AtLBD1* and *AtLBD11*. The closest *Citrus sinensis* homolog of these Arabidopsis genes, *CsLOB1*, was found to function as a disease susceptible gene in citrus bacterial canker, a disease caused by multiple *Xanthomonas* species (Hu et al., 2014). Moreover, *CsLOB2* and *CsLOB3*, belonging to the same clade as *CsLOB1*, were found to have a similar role as *CsLOB1* in citrus bacterial canker (Zhang et al., 2017). Another putative disease susceptible gene might be *VviLBDIi2*, which was down-regulated in partially and completely

resistant plants derived from *Muscadinia rotundifolia* when inoculated with *Plasmopara viticola*. Interestingly, *VviLBDIi2* presented in its promoter *cis*-acting elements involved in salicylic acid responsiveness, a hormone known to be involved in response to biotrophic pathogens (Agudelo-Romero et al., 2015). Furthermore, *VviLBDIa1*, *VviLBDIf13* and *VvLBDIIb1* seems to be involved in early response to biotrophic fungus, since were up-regulated in response to *E. necator* infection at green stage. *VviLBDIa1* co-expressed with genes associated with biotic stress, such as genes encoding anthranilate N-benzoyltransferase and chalcone and stilbene synthase. *VviLBDId4* was up-regulated in powdery mildew infected berries at *véraison,* indicating that might be involved in later responses.

The involvement of grapevine LBD genes in response to biotic stress was also noticed for Bois noir disease. *VviLBDIc3* showed down-regulation in inflorescences presenting Bois noir disease, in grape berries after long exposure to *B. cinerea*, cold and ABA treatment, which could suggest that some LBD genes may be simultaneously modulated by abiotic and biotic stress conditions.

3.5. Conclusions

LOB domain (LBD) transcription factors families have been characterized in several plant species and shown to participate in the regulation of developmental programs and stress responses. Nevertheless, the role of LBDs in fruit ripening has been poorly documented. Modulation of *LBD* genes expression during grape berry development and ripening indicates that these processes may be under regulation of LBD transcription factors. In addition, several grapevine *LBD* genes bared *cis*-elements in their 5′ regulatory region associated with defense and hormonal regulation, which together with expression and co-expression analyses supports their involvement in the abiotic and biotic stress response mechanisms. Candidate genes were identified that exhibit broad response to stress (e.g., *VviLBDIc3)* or could be involved in grape ripening and grape defense (e.g., *VviLBDId6).* Altogether this data may be used for functional characterization of genes and ulterior improvement of fruit quality traits and resilience to abiotic and biotic stresses.

3.6. Methods

3.6.1. Identification of *LBD* genes.

Genes previously identified as encoding LOB domain proteins in Grimplet *et al*.30 were blasted (blastp and tblastn) against the grapevine genome 12Xv.2

(https://urgi.versailles.inra.fr/Species/Vitis/Data-Sequences/Genome-sequences), the non-redundant list of genes in Grimplet et al. (2012), the NCBI Refseq (both remapped on the 12Xv2 of the genome assembly) and the COST annotation gene set available at the ORCAE website (http://bioinformatics.psb.ugent.be/orcae/). Results from different analyses were manually cross-checked to identify new potential loci corresponding to *LBD* genes in the grapevine genome. The UGene software (Okonechnikov et al., 2012) was used to design the gene models on the grapevine genome and test their structure.

3.6.2. Gene structure analysis.

The potential coding DNA sequences (CDS) were blasted (blastx) against the NCBI public database to compare the structures with other known *LBD* genes in other species and the NCBI Refseq predictions of the grapevine genes. When discrepancies were observed, gene models were corrected using the UGene software. Loci bearing non-functional genes were eliminated from the list. A GFF file with the *LBD* genes was designed, uploaded into the IGV software and the RNAseq data available on flowers in the laboratory were used to double-check the exon structure of the genes.

3.6.3. Promoter analysis.

Promoter *cis*-acting regulatory elements within 1.5 kb of the upstream sequence from the ATG initial codon of each grapevine *LBD* gene were analyzed with PlantCARE software (Lescot et al., 2002) (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/). Analysis of transcription factor binding sites (TFBSs) of the 3 kb upstream sequence region of the initial codon was also performed using the Plant Promoter Analysis Navigator (PlantPAN) software (Chang et al., 2008) (http://plantpan2.itps.ncku.edu.tw/).

3.6.4. Enrichment of *cis*-regulatory elements.

Motif analysis of known and *de novo* motifs was performed using Homer v4.9 (Heinz et al., 2010) (http://homer.ucsd.edu/homer/motif/). With this end, grapevine promoter sequences (2.5 kb upstream of the coding sequence) of *LBD* genes were retrieved from Regulatory Sequence Analysis Tools (RSAT, http://foresta.eead.csic.es/rsat/). Additionally, in order to prevent overlapping between neighboring genes, noorf option was performed.

3.6.5. Sequence alignment and phylogenetic analysis.

Sequence information on previously reported LOB domain proteins of *A. thaliana* was retrieved from the Arabidopsis Information Resource (https://www.arabidopsis.org/).

Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013). Multiple sequence alignment was inferred using MUSCLE (Edgar, 2004). The evolutionary history was inferred by using the Maximum Likelihood method based on the JTT matrix-based model (Jones et al., 1992). The bootstrap consensus tree inferred from 100 replicates was taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 30% of bootstrap replicates were collapsed. Initial trees for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value. The coding data was translated assuming a standard genetic code table. All positions with less than 95% site coverage were eliminated. The genes were named according to Grimplet and co-workers (2014) based on the distance homology with Arabidopsis genes. The alignment file between Arabidopsis and grapevine sequences was uploaded to the Jalview and UGene software for manual adjustment of the alignment and manual motif editing. Motifs were fagged and labeled for the grapevine genes; additional motifs of high homology were also identified (at least 50% homology within the members of the subfamily on at least 10 amino acids) among grapevine sequences.

3.6.6. Expression analysis.

Expression data were retrieved from 3 different microarray platforms (Affymetrix Genchip (16k probesets), GrapeGen (21k probesets), Vitis Nimblegen array (29k probesets) and from our in-house RNAseq projects. Data normalization was performed on all the array of each platform (RMA normalization). After retrieving the values for the probesets corresponding to each gene, the values for the 3 or 4 replicates of the same condition were averaged to obtain a total of up to 256 conditions (organ, cultivar, treatment, platform) for the genes present in all platform. Based on expression data of the grapevine gene expression atlas (Fasoli et al., 2012), a plant ontology ID was attributed to each gene if expression intensity in a tissue was above a defined threshold of absolute log2 value of 8 or absolute value of 256. The same data were used for the co-expression analysis with the whole set of genes available on the Nimblegen platform. Hierarchical clustering with Pearson correlation as metric and average linkage cluster method was performed. Genes considered as having the same profile should present a distance threshold between each other lower than of 0.2. For further evaluation of gene expression samples corresponding to several stages of grapevine development and ripening and several abiotic and biotic stress conditions were used (Agudelo-Romero et al., 2015; Albertazzi et al., 2009; Carbonell-Bejerano et al., 2013; Carvalho et al., 2011; Cramer et al., 2007; Deluc et al., 2007;

Díaz-Riquelme et al., 2012; Espinoza et al., 2007; Fasoli et al., 2012; Fortes et al., 2011; Fung et al., 2008; Grimplet et al., 2007; Lijavetzky et al., 2012; Lund et al., 2008; Pilati et al., 2007; Pontin et al., 2010; Sreekantan et al., 2010; Tattersall et al., 2007; Tillett et al., 2011; Vega et al., 2011). Heat maps were performed with the ComplexHeatmap R package (https://github.com/jokergoo/ComplexHeatmap). RNAseq results obtained in Chapter 2 were also used for gene expression evaluation during ripening and in response to powdery mildew.

3.6.7. Sequence comparison among diverse plant species.

We performed a sequence comparison using the LBD genes from 33 plant species (*Arabidopsis thaliana, Brassica rapa, Carica papaya, Teobroma cacao, Gossypium raimondii, Eucalyptus grandis, Citrus clementina, Manihot esculenta, Ricinus communis, Populus trichocarpa, Linum usitatissimum, Malus domestica, Pyrus bretschneideri, Prunus persica, Fragaria vesca, Cicer arietinum, Glycine max, Medicago truncatula, Citrullus lanatus, Cucumis sativus, Solanum lycopersicum, Utricularia gibba, Nelumbo nucifera, Hordeum vulgare, Triticum aestivum, Oryza sativa subsp. indica, Phyllostachys heterocycla, Sorghum bicolor, Zea mays, Musa acuminata, Phoenix dactylifera, Picea abies*) retrieved at http://planttfdb.cbi.pku.edu.cn. We identified orthologous genes in genomes from the thirty-three species following what was performed in Jaillon et al. (2007). Each pair of predicted gene sets was aligned with the BLASTp algorithm, and alignments with an e-value lower than 1e-20 and sequence homology higher than 40% were retained. If a comparison is above that value, the two genes were considered homologs. Two genes, A from Vitis genome (GV) and B from a given species genome (GX), were considered orthologs one-to-one if B was the best match for gene A in GX and A was the best match for B in GV. A phylogenetic tree was constructed with the LBD genes from these species with the same parameters as before. A Ka/Ks analysis was performed using the Ka/Ks calculation tool (http://services.cbu.uib.no/tools/kaks) on all the orthologs detected in the species for each grapevine gene with the default parameters.

3.7. Supplementary Data

Supplementary Table S3.1. Complete annotation of the grapevine *LBD* genes and ka/ks analysis.

Supplementary Table S3.2. Complete *cis*-regulatory elements analysis of *VviLBD* genes promoters with PlanCARE software.

Supplementary Table S3.3. Complete analysis of transcription factor binding sites analysis in *VviLBD* genes promoters with PlantPAN software.

Supplementary Table S3.4. Motifs enrichment data. Identification of known and *de novo* motifs using Homer software. List of *VviLBD* genes containing the *de novo* motif (GGTTGAATACAC); the scan was performed allowing up to two substitutions.

Supplementary Table S3.5. List of co-expressed genes with *LBD* genes.

Supplementary Figure S3.1. Exon-intron structure of the 50 grapevine *VviLBD* genes. Exons are represented by yellow boxes and introns by black lines. The majority of *VviLBD* genes presented two exons (37 genes). Gene structures of the *VviLBD* genes were generated with the GSDS 2.0 (http://gsds.cbi.pku.edu.cn/).

Supplementary Figure S3.2. Grapevine LBD proteins alignment.

Supplementary Figure S3.3. Molecular phylogenetic analysis by Maximum Likelihood method between grapevine and 33 plant species.

Supplementary Figure S3.4. Distribution of *cis*-regulatory elements in the *VviLBD* promoter regions. Bar graph depicts the total number of genes for each *cis*-element motif. Several regulatory motifs were identified as associated with light regulation, defense and stress responses, and hormonal regulation.

Supplementary Figure S3.5. Distribution of transcription factor binding sites in the *VviLBD* promoter regions. Bar graph depicts the total number of genes for each transcription factor binding site motifs. Several transcription factor binding sites were identified as associated with developmental processes and with biotic and abiotic stress responses.

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Chapter 4

CRISPR-Cas9 mediated mutagenesis of SlGRAS10 in tomato plants

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Authors' Contributions: Ana Margarida Fortes designed and directed the study. Diana Pimentel performed the cloning and plant transformation experiments under the supervision of Antonio Granell who also provided funding for these experiments. D.P. wrote the chapter. A.M.F and A.G revised the chapter.

4.1. Abstract

GRAS transcription factor family has been reported as involved in multiple processes, such as signaling, plant development, and stress response. The role of GRAS proteins in fruit ripening is poorly studied; nevertheless, previous broad expression pattern analysis revealed differential expression among ripening stages in some fruit plants. Additionally, RNAseq data obtained as described in Chapter 2 revealed that several GRAS genes were modulated with the onset of ripening and in response to powdery mildew infection.

Tomato (*Solanum lycopersicum*) *SlGRAS10* and its grapevine ortholog *VviPAT6* were previously suggested as putative regulators of fruit ripening in both climacteric and non-climacteric plants. In this chapter, preliminary studies were developed to functionally characterize the *SlGRAS10* gene in tomato through reverse genetics. Tomato was chosen due to the fact that genetic transformation in this species is less time-consuming than in grapevine. CRISPR-Cas9 technology was applied to the targeted mutagenesis of *SlGRAS10* in *Solanum lycopersicum* cv. MoneyMaker. Two gRNAs were designed and assembled with the Cas9 endonuclease and the selection marker. Tomato cotyledons were transformed with the final constructs through *Agrobacterium*-mediated stable transformation. Two over-expression constructs were also assembled under the control of CaMV 35S promoter, a constitutive promoter, and E8, a fruit-specific promoter. Sequence and phylogenetic analysis confirmed the presence of the GRAS domain in the SlGRAS10 protein sequence. Promoter analysis was also performed, revealing several regulatory motifs associated with hormonal regulation, stress response, and several Myb binding sites.

4.2. Introduction

Transcription factors are proteins containing domains that interact with DNA-regulatory sequences (enhancers and silencers), being critical pieces in the modulation of gene expression in multiple processes, such as plant development and response to external biotic and abiotic stimuli. The GRAS transcription factor family have been described as involved in the regulation of plant development and signaling, as well as in response to stress (reviewed by Bolle, 2016). The name of this family was originally from the three initially identified members, GAI (gibberellic acid insensitive), RGA (repressor of GA1) and SCR (scarecrow) (Bolle, 2004; Pysh et al., 1999). Typically, GRAS proteins have 400–770 amino acid residues and show sequence homology to each other in their C-termini, where five conserved motifs are located: leucine heptad repeats I (LHR I), VHIID, leucine heptad repeats II (LHR II), PFYRE, and SAW (Pysh

et al., 1999; Tian et al., 2004). On the other hand, the N-terminal is highly variable, both in sequence and length, and probably responsible for the functional specificity (Tian et al., 2004). Phylogenetic analyses have shown that the GRAS proteins family can be grouped into several clades, such as DELLA, SCR, LAS, HAM, PAT1, LISCL, SHR, SCL3, and SCL4/7.

Several GRAS proteins have been functionally characterized in diverse plant species, and are involved in several biological processes, including gibberellin signaling (Peng et al., 1997; Yoshida et al., 2014), phytochrome A signal transduction (Bolle et al., 2000; Torres-Galea et al., 2013), root development (Di Laurenzio et al., 1996; Helariutta et al., 2000; Pysh et al., 1999), axillary meristem initiation (Greb et al., 2003; Li et al., 2003), and shoot meristem maintenance (Stuurman et al., 2002). Several studies also reported the involvement of GRAS members in response to both biotic (Czikkel and Maxwell, 2007; Mayrose et al., 2006) and abiotic stresses (Liu et al., 2020, Ma et al., 2010; Yang et al., 2011), and also symbiotic interactions (Gobbato et al., 2012; Hartmann et al., 2019; Hirsch et al., 2009). Based on expression analysis, GRAS genes were shown to be modulated in response to pathogen infection, such as *Botrytis cinerea* and/or powdery mildew (Chapter 2; Grimplet et al., 2016). Some of these responsive genes belong to the PAT clade, including the *VviPAT6* and *VviPAT4* that were up-regulated in response to infection with *B. cinerea* and both *B. cinerea* and powdery mildew, respectively (Chapter 2; Grimplet et al., 2016).

Arabidopsis PAT1 was the first member of the PAT1 subfamily to be characterized and is involved in phytochrome signal transduction (Bolle et al., 2000). Nevertheless, other PAT1 members from different species were associated with different functions. *VaPAT1* was shown to be involved in abiotic stress responses (Yuan et al., 2016). Two rice members of the PAT1 clade, OsCIGR1 and OsCIGR2, were shown to be induced upon N-acetylchitooligosaccharide elicitor perception acting as transcriptional regulators of the elicitor-induced defense responses (Day et al., 2004, 2003). Common bean SIN1 (Scarecrowlike13 Involved in Nodulation 1) was found to participate in lateral root elongation and root symbiosis through posttranscriptional silencing using RNA interference (Battaglia et al., 2014).

Previous studies on identification and broad expression pattern analysis of the *GRAS* genes in grapevine revealed that *VviGRAS* genes showed differential expression among berry ripening stages suggesting a key role in the grape berry development (Grimplet et al., 2016). Moreover, *VviPAT6* revealed an expression pattern consistent with their involvement in berry ripening along with its orthologs in tomato fruits, *SlGRAS10*, suggesting that these genes could have conserved functions in transcriptional regulation of ripening not only in non-climacteric as well as in climacteric fruits (Grimplet et al., 2016; Huang et al., 2015). Very little is known

about the involvement of GRAS proteins in the ripening process. In strawberry, a non-climacteric fruit, *FaSCL8* expression was shown to be induced in the receptacle coincident with ripening and to reach higher expression at the red ripe stage (Pillet et al., 2015). Moreover, *FaSCL8* gene silencing led to lower transcript accumulatio of *PAL*, *CHS*, *CHI*, *F3H*, *UFGT*, and *MYB10* but increased accumulation of F3'H and ANR. *MYB10* and *MYB9/11* were indicated as possible targets or interactors of FaSCL8, suggesting that FaSCL8 might regulate the flavonoid pathway through modulation of Myb transcription factors expression affecting the cyanidin-pelargonidin balance (Pillet et al., 2015). The expression of *FaSCL8* was stronger in strawberry receptacle compared with to vegetative tissues, reinforcing the putative involvement of FaSCL8 in receptacle fruit ripening (Medina-Puche et al., 2016). In bilberry, also a non-climacteric fruit, *VmSCL8* expression was increased in ripe fruits and up-regulated by ABA, suggesting an involvement in fruit ripening, mainly at later stages, and possibly regulated by ABA (Karppinen et al., 2018). ABA has been reported as a key positive regulator of ripening in non-climacteric fruits, such as grape berries and strawberry (Fortes et al., 2015; Fuentes et al., 2019). In grape berries, ABA was shown to accumulate at the *véraison* stage (Coelho et al., 2019).

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) - CRISPR-associated protein 9 (Cas9) system is nowadays popularly used to targeted mutagenesis. CRISPR-Cas9 technology is based on the imperfect double-strand DNA break repair that creates INDELs in the target region, and when located in an open reading frame, can create frame-shift mutation leading to loss-of-function. CRISPR-Cas9 technology relies on guideRNAs (gRNAs) that recognize the target region and directs the Cas9 endonuclease to create the double-strand DNA breaks. The CRISPR technology has been adapted to modular cloning strategies, such as the GoldenBraid cloning system, facilitating the application to functional studies and allowing the combination of several gRNAs targeting more than one gene (Vazquez-Vilar et al., 2016). CRISPR-Cas9 system has been successfully applied in many plant species, including tomato (*Solanum lycopersicum*). Tomato is an excellent plant model to study climacteric fleshy fruit development not only due to its short life cycle compared to other fruit plants and ease manipulation but also its importance in the human diet and economy.

In this chapter, preliminary studies were developed to characterize the *SlGRAS10* gene in tomato through reverse genetics. Two gRNAs were selected and assembled with the Cas9 endonuclease and the kanamycin resistance as a selection marker. A stable transformation strategy was selected to obtain mutant tomato plants. Two over-expression constructs were also assembled under the control of CaMV 35S promoter, a constitutive promoter, and E8, a fruit-

specific promoter. Sequence and phylogenetic analysis were also performed, confirming that SlGRAS10 has a conserved GRAS domain at the C-terminal and belongs to the PAT1 clade. Further studies are being conducted in our group to characterize the *SlGRAS10* ortholog in grapevine, the *VviPAT6*.

4.3. Results

4.3.1. Sequence and Phylogenetic analysis

SlGRAS10 (Solyc03g025170.1.1) gene is located at chromosome 3 of the *Solanum lycopersicum* genome. *SlGRAS10* gene is intronless, and the coding DNA sequence has 1896 nucleotides translated into a polypeptide with 631 amino acid residues. SlGRAS10 protein has a theoretical molecular weight of 69.1 kDa and a predicted isoelectric point of 6.20. The total number of negatively (Asp and Glu) and positively charged residues (Arg and Lys) were 60 and 56, respectively. A phylogenetic tree was constructed to determine the evolutionary relationship of *SlGRAS10* with other GRAS applying the maximum likelihood method to an alignment that included the seven highest hits retrieved with the BLASTp algorithm from tomato, grapevine, and Arabidopsis. Phylogenetic analysis confirmed that the *SlGRAS10* belongs to the PAT1 subfamily, close to *SlGRAS9* (Solyc06g036170.1.1), *AtSCL8* (*Arabidopsis thaliana SCARECROW-LIKE 8*, AT5G52510.1), and *VviPAT6* (VIT_16s0050g00950) genes (Figure 4.1). PAT subfamily could be divided into two branches, the one with close homology to the *AtPAT1* and the other with *AtSCL1* and *AtSCL8*. The evolutionary distance between *AtSCL8* homologs was estimated, and *SlGRAS10* shared 69.2% of identity with *SlGRAS9*, 58.9% with *VviPAT6*, and 53.2% with *AtSCL8*.

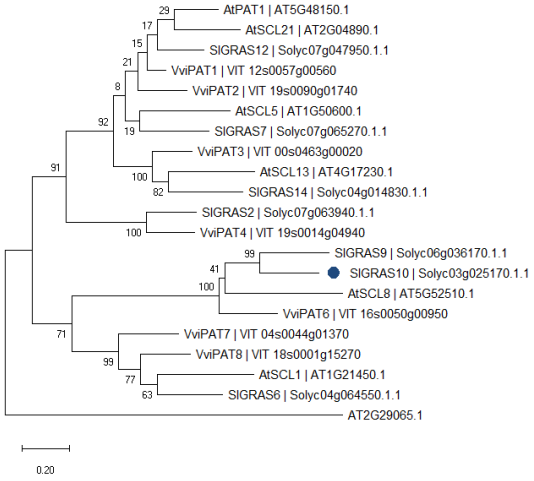

Figure 4.1 - Phylogenetic analysis with GRAS sequences. Phylogenetic analysis was performed with MEGAX software applying the Maximum Likelihood method based on a JTT matrix-based model, with 1000 replicates**.**

The deduced amino acid sequence of SlGRAS10 contains the typical GRAS domain in the C-terminus, where the most common motifs were found, namely LHRI, VHIID, LHRII, PFYRE, and SAW motifs (Bolle, 2004) (Figure 4.2). LHR motifs have been suggested as essential in homo- and hetero-dimerization (Xue et al., 2015; Zentella et al., 2007).

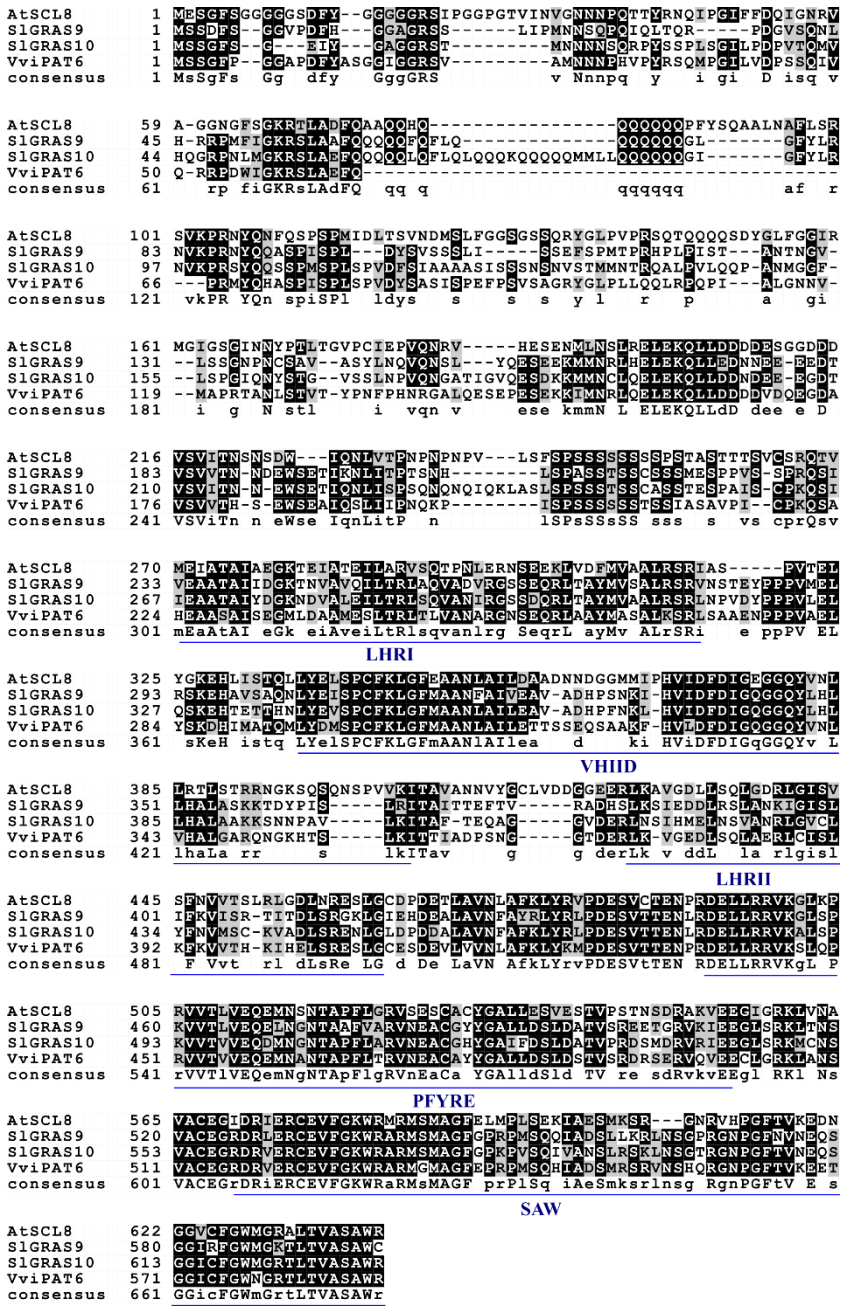

Figure 4.2 - Alignment of the amino acid sequences of Arabidopsis SCL8 with SlGRAS9, SlGRAS10, and VviPAT6. Conserved sequences are shaded, and gaps are indicated as dashes in the sequence.

Secondary structure analysis of SlGRAS10 protein was predicted by PSIpred programs and showed that SlGRAS10 contains 20 α-helixes and 11 strands, within 16 α-helixes and 10 strands located at the GRAS domain (Figure 4.3). Since the GRAS domain has high homology

within the GRAS proteins, the predicted secondary structure of the SlGRAS10 GRAS domain (Figure 4.3) is similar to the previously reported crystal structures of the GRAS domain of SHR-SCR heterodimer and SCL7 homodimer (Hirano et al., 2017; Li et al., 2016), despite they belong to different clades. The rice SCL7 GRAS domain was reported to contain an eight-stranded mixed β-sheet (B1-B9) with an accessory small β-strand B6 and 12 α-helices (A1-A12) as well as five 3_{10} helices, forming a Rossmann fold core subdomain and an additional cap subdomain (Li et al., 2016). SHR GRAS domain contained 14 α- and three 3_{10} helices for SHR and nine β-strands, forming an α-helical cap at the N-terminal and α/β core subdomain (Hirano et al., 2017).

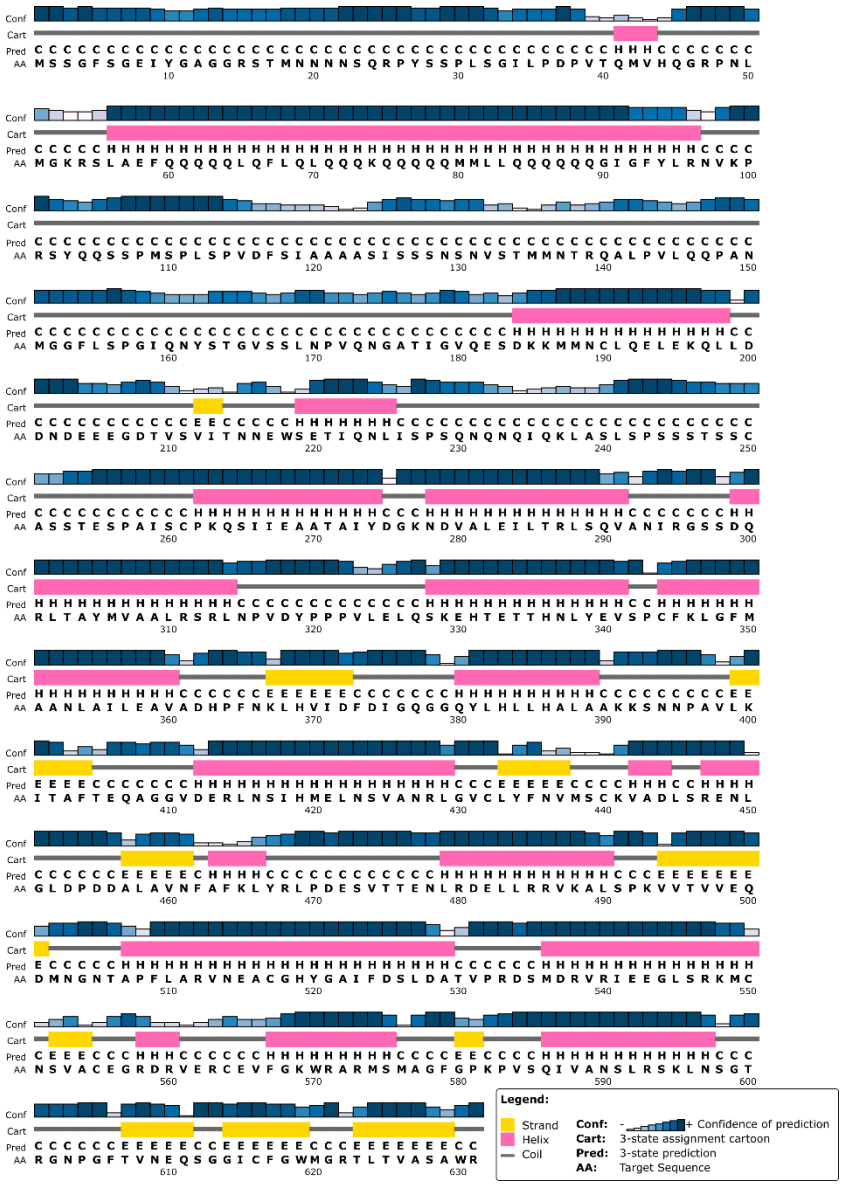

Figure 4.3 - Predicted secondary structure of SlGRAS10 using the PSIpred program (http://bioinf.cs.ucl.ac.uk/psipred/).

4.3.2. Transcript levels in developing fruit and other tissues

To evaluate the expression patterns of the *SlGRAS10* gene in tomato tissues and during fruit ripening, the absolute values of gene expression in different organs (root, leaf, flower, fruit, and seed) were retrieved from public data (TomExpress database) for both MicroTom and Heinz 1706 varieties (Figure 4.4A and B). *SlGRAS10* was expressed in the different tissues with higher levels at flower and fruit tissues (Figure 4.4A and B). In both varieties, *SlGRAS10* showed increased expression during ripening, suggesting an involvement in the ripening process (Figure 4.4A and B). During ripening in MicroTom fruits, the highest expression was observed at the red ripe stage DPA (44 DPA). Nevertheless, Heinz 1706 fruits reached the highest expression at the breaker stage (Figure 4.4A and B).

Additionally, *SlGRAS10* expression was also analyzed in tomato fruits at the Red Ripe stage from three different varieties (MS4, TRVA002 'Valenciano', and TRVA003 'Penjar') and two hybrids (TRVA002 x MS4 and TRVA003 x MS4) (Figure 4.4C). MS4 is a breeding line that carries a *rin* mutation and is characterized by impaired ripening. The *rin* mutation is a gain-of-function mutation that results in the production of a chimeric protein due to a delection between two MADS-box transcription factor enconding genes: MADS-RIN (RIPENING INHIBITOR) and MADS-MC (MACROCALYX) (Ito et al., 2017; Vrebalov et al., 2002). 'Penjar' variety is characterized by a longer shelf-life, due to *alc (alcobaça)* mutation, and by reduced fruit size. The *alc* mutation is an allele of the *non-ripening* (*nor*) with a weaker effect on ripening as a result of a single amino acid substitution on the coding region of the NAC-NOR gene (Casals et al., 2012). 'Valenciano' is a traditional Spanish variety with no delayed ripening. Red ripe 'Valenciano' fruits presented a lower expression of *SlGRAS10* compared to MS4, and 'Penjar' and expression level was increased when crossed with MS4 variety (Figure 4.4C). However, its expression was reduced on MS4 and 'Penjar' hybrid (Figure 4.4C). Results suggest that *rin* and *alc* mutation separately had a positive effect on *SlGRAS10* expression. Further analysis will be essential to understand the interaction between *SlGRAS10* and other ripening-related genes.

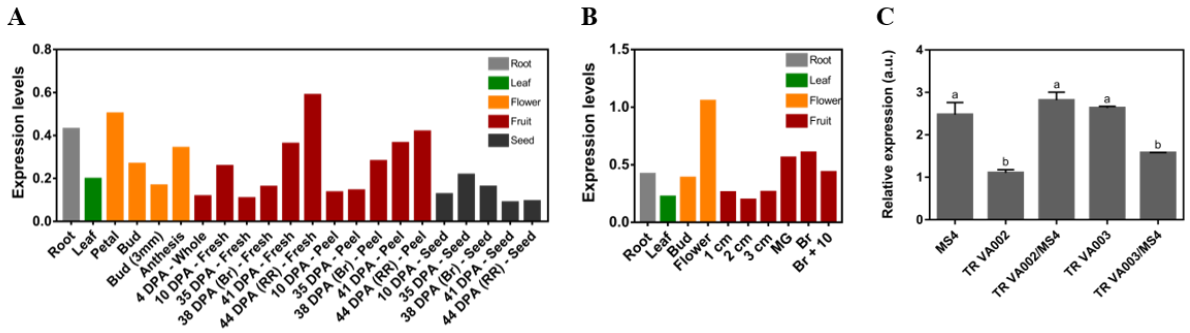

Figure 4.4 – Expression analysis of the *SlGRAS10*. *SlGRAS10* expression levels in different plant tissues and during fruit ripening in **(A)** MicroTom and **(B)** Heinz 1706 varieties. Expression data was retrieved from the TomExpress database (http://tomexpress.toulouse.inra.fr/). **(C)** *SlGRAS10* expression on red ripe tomato fruits from three varieties (MS4, TRVA002 'Valenciano' and TRVA003 'Penjar') and two hybrids (TRVA002 x MS4 and TRVA003 x MS4). Gene expression analysis performed by real-time PCR. One-way ANOVA and Tukey's multiple comparisons test were performed to indicate statistical differences.

4.3.3. Analysis of promoter sequence

Cis-acting regulatory elements in the promoter region are essential for the regulation of gene expression (Hernandez-Garcia and Finer, 2014). In *silico* analysis of the *SlGRAS10* promoter region was carried out using PlantCARE database to predict *cis*-acting elements and revealed several regulatory motifs associated with light regulation (Box I, BOX4 and LAMP-element), heat stress responses (HSE), biotic stress (Box-W1), hormonal regulation including ethylene (ERE) and salicylic acid (TCA-element), and regulatory elements associated with tissue-specific expression (CAT-box, Skn-1_motif, RY-element), in addition to the core promoters, TATA-box and CAAT-box, present in all promoter regions (Table 4.1). Several MYB binding sites were detected, including the MBSI element, which is involved in the regulation of flavonoid biosynthesis, MBS, related to drought inducibility, and MRE, involved in light responsiveness (Table 4.1).

Moreover, several transcription factors binding sites were also recognized related to several developmental processes and stress responses, including AP2/ERF, WRKY, MADS-box, Myb, NAC, bZIP, LBD, and bHLH (Table 4.2). MADS-box transcription factors, where RIN protein is included, are involved in a wide variety of plant processes, including fruit development. Additionally, one GRAS binding site was identified in the *SlGRAS10* promoter region (Table 4.2).

Table 4.1 - *Cis*-regulatory elements identified in the *SlGRAS10* promoter region (2500bp upstream of the start codon) analyzed using PlantCARE software (Lescot et al., 2002).

***Cis*-element**	**Number of motifs**	***Cis*-element**	**Number of motifs**	***Cis*-element**	**Number of motifs**
AAGAA-motif	1	GA-motif	1	RY-element	1
ARE	2	GT1-motif	1	Skn-1_motif	3
ATCT-motif	1	GTGGC-motif	1	STRE	1
AT~TATA-box	5	HSE	2	TATA	2
Box 4	4	I-box	2	TATA-box	67
Box I	3	LAMP-element	2	TCA-element	2
Box III	1	MBS	2	W box	3
Box-W1	2	MBSI	1	WRE3	1
3-AF1 binding site	1	MRE	1	WUN-motif	1
CAAT-box	43	MYB	2	circadian	2
CAT-box	1	MYB recognition site	1	5UTR Py-rich stretch	3
CCAAT-box	1	MYB-like sequence	1		
ERE	7	MYC	6		

Table 4.2 - Transcription factor binding sites (TFBS) in the *SlGRAS10* promoter region (2500bp upstream of the start codon) were analyzed using PlantPAN software (Chow et al., 2019).

TF binding site	**Number of motifs**	**TF binding site**	**Number of motifs**	**TF binding site**	**Number of motifs**	**TF binding site**	**Number of motifs**
Amylase-like proteins	48	FAR1	7	MADF; Trihelix	33	NF-YB; NF-YA; NF-YC	96
AP2	1	GATA	23	MADS box	73	PsaH	1
AP2; B3; RAV	11	GeBP	1	MADS box; MIKC	5	SBP	53
AP2; ERF	144	GRAS	1	MYB	26	Sox; YABBY	7
ARID	19	GRF	2	Myb/SANT	60	SRS	5
ARID; Sox	22	HB-PHD	6	Myb/SANT; ARR-B	4	Storekeeper	2
AT-Hook	192	HD-ZIP	20	Myb/SANT; G2-like	19	TBP	39
Aux/IAA	2	Homeodomain; bZIP; HD-ZIP	25	Myb/SANT; MYB	101	TCP	72
B3	65	Homeodomain; bZIP; HD-ZIP; WOX	14	Myb/SANT; MYB; ARR-B	20	TCR	28
bHLH	36	Homeodomain; HB-PHD	1	Myb/SANT; MYB; G2-like	1	TCR; CPP	84
bZIP	53	Homeodomain; HD-ZIP	83	Myb/SANT; MYB-related	143	Trihelix	87
C2H2	52	Homeodomain; TALE; TALE	15	MYB; ARR-B	2	VOZ	3
C3H Zinc finger	15	HSF	6	MYB; G2-like	3	WOX	11
CG-1; CAMTA; CAMTA	10	LBD	3	MYB; MYB-related	1	WRC; GRF	1
Dof	322	LEA	4	MYB-related	32	WRKY	135
E2F	4	LIM	2	NAC	17	YABBY	9
EIN3; EIL	7	MADF	7	NAC; NAM	42	ZF-HD	149

4.3.4. CRISPR/Cas9 technology application to the functional characterization of the *SlGRAS10* gene.

Target loss-of-function mutation of the *SlGRAS10* gene using CRISPR/Cas9 technology was applied to investigate its biological role in tomato ripening through reverse genetics. Potential single guide RNAs (sgRNAs) with twenty nucleotides targeting the *SlGRAS10* coding region were identified using the Benchling's CRISPR tool. The two sgRNA sequences with a G at the 5' end and with the highest on- and off-target scores were selected for further tomato mutation (Table 4.3). The on-target score refers to the cleavage efficiency of the Cas9, and the off-target score represents the inverse probability of the sgRNA to target other regions of the genome (Doench et al., 2016; Hsu et al., 2013). sgRNA_1 targeted the beginning of the coding sequence at the 37 bp. sgRNA_2 was located at 909 bp downstream the start codon on the antisense strand, targeting the GRAS domain at the C-terminus of the protein sequence (Table 4.3). Since the Cas9 enzyme cuts both DNA strands forming a blunt end, the PAM sequence can be located on either the sense or the antisense strand. sgRNAs were assembled with the U6-26 promoter and the scaffold RNA using the GoldenBraid 3.0 cloning system to build the complete gRNA expression cassette (Vazquez-Vilar et al., 2017, 2016).

Table 4.3 - Single guide RNAs (sgRNAs) selected targeting the *SlGRAS10* gene. Scores retrieved from Benchling's CRISPR tool (https://www.benchling.com) based on algorithms developed by Doench et al. (2016) and Hsu et al. (2013).

sgRNA	Sequence	PAM	Position	Strand	On-target score	Off-target score
sgRNA_1	GCGAGATTTACGGCGCCGGT	GGG	37	+	51.8	49.8
sgRNA_2	GTTCAAGCGCGACCGTAAAG	CGG	909	-	75.6	49.8

Subsequently, sgRNA expression cassettes were assembled with hCas9-encoding transcription unit and with selection marker that confers kanamycin resistance (NptII) to form the final construct (Figure 4.5A and B).

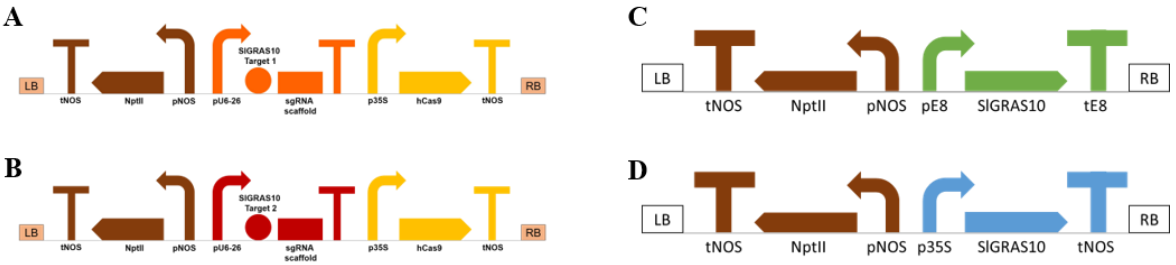

Figure 4.5 - Schematic representation of the constructions used for *SlGRAS10* functional characterization. Guide RNA expression cassettes targeting two different sites of the *SlGRAS10* gene. **(A)** sgRNA_1; **(B)** sgRNA_2. Over-expression cassettes under the control of two promoters: **(C)** E8 promoter, fruit-specific; and **(D)** 35S promoter, constitutive Constructs were assembled using GoldenBraid 3.0 cloning system (Vazquez-Vilar et al., 2017).

Tomato var. Moneymaker cotyledons were transformed with *Agrobacterium tumefaciens* LBA4404 (Figure 4.6A). Explants were placed on *callus* induction medium of MS agar with hormones (IAA 1 mg/L and *trans*-zeatin 0.75 mg/L) and antibiotics (kanamycin 100 mg/L, carbenicillin 400 mg/L and timentin 150 mg/L). *Calli* were then transferred to shoot induction media supplemented with IAA (0.1 mg/L) and *trans*-zeatin (0.75 mg/L) (Figure 4.6B). Shoots developed from *callus* were elongated in shoot elongation media (*trans*-zeatin 0.1 mg/L) (Figure 4.6C), when reached around 2 cm in height were cut off and transferred to rooting media supplemented with IBA (0.2 mg/L) to obtain whole plants (Figure 4.6D). The next step was the evaluation of the CRISPR-Cas9 action through the amplification and sequencing of the target region, performed by Flávio Soares in the scope of his PhD thesis.

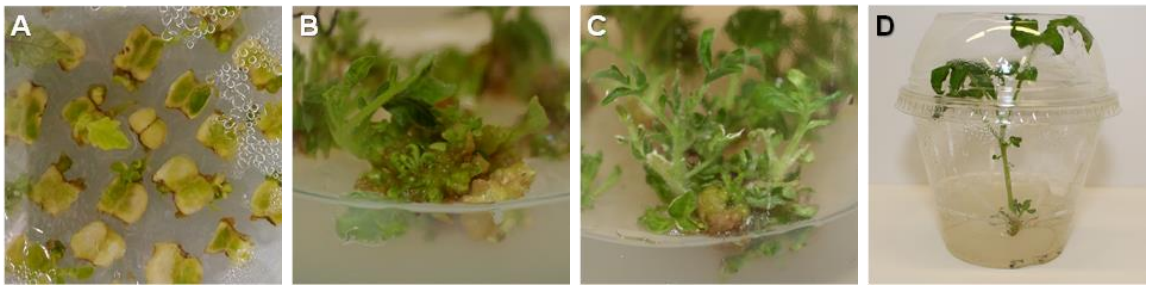

Figure 4.6 - Stable transformation of tomato var. MoneyMaker with Agrobacterium tumefaciens. (A) *Calli* induction; (B) Shoot induction; (C) Shoot elongation, and (D) Rooting and plant development.

4.3.5. Construct design for *SlGRAS10* overexpression

To complement the functional characterization, *SlGRAS10* over-expression constructs were designed and assemble for further tomato transformation. *SlGRAS10* was amplified by PCR from the genomic DNA isolated from *S. lycopersicum* var. Moneymaker and sequenced after domestication in pUPD vector. *SlGRAS10* gene was then assembled under control of cauliflower mosaic virus 35S (CaMV 35S) and E8 promoters with the respective terminators (Figure4.5C and D). The CaMV 35S promoter efficiently drives the expression of the selected gene in plant cells (Jani et al., 2002); however, this promoter does not allow either tissue or developmental stage specificity. E8 promoter has been identified in tomato as a fruit-specific promoter. E8 promoter has at least two main regulatory regions: one located between –2181 to –1088 upstream E8 gene that contains several *cis*-acting elements responsive to ethylene and other downstream the previous region that is not responsive to ethylene but is enough for ripening-specific gene expression in the absence of ethylene (Deikman et al., 1992). Final vectors were mobilized into *A. tumefaciens* LBA4404 by electroporation for further tomato stable transformation performed by Flávio Soares in the context of his Ph.D. thesis.

4.4. Discussion and Future Perspectives

GRAS proteins represent an important family of regulatory proteins widely identified in the plant kingdom. Characterized GRAS proteins have been associated with plant development and signaling, as well as stress responses (Bolle, 2016). However, several GRAS proteins are still uncharacterized, and more functional studies might reveal other functions. Recent genome-wide studies suggested the involvement of grapevine *VviPAT6* and tomato *SlGRAS10* orthologs in the onset of ripening in both non-climacteric and climacteric plants (Grimplet et al., 2016). In order to explore the putative involvement of the SCL8 proteins in ripening, CRISPR-Cas9 technology, as well as overexpression studies, are being applied to functional characterization of tomato *SlGRAS10*. Tomato is an important model species for functional genomics, due to its short life cycle, ease manipulation and transformation, medium-size genome, and the availability of the genetic and phenotypic data.

Sequence analyses confirmed that SlGRAS10 (Solyc03g025170.1.1) presents a highly conserved GRAS domain in the C-terminal region, where could be identified the five central conserved motifs (LHRI, VHIID, LHRII, PFYRE, and SAW) previously described in *Arabidopsis thaliana* and *Oryza sativa* (Pysh et al., 1999; Tian et al., 2004). SlGRAS10 was grouped at the AtSCL8 branch of the PAT1 subfamily, together with the SlGRAS9 and VviPAT6.

Expression data retrieved from public databases demonstrated an accumulation of the *SlGRAS10* transcripts during ripening in MicroTom and Heinz 1706 varieties, reinforcing the putative involvement in fruit ripening (Figure 4.4A and B). Strawberry (*Fragaria vesca*) and bilberry (*Vaccinium myrtillus*) homologs of AtSCL8 were also suggested to be involved in fruit ripening (Karppinen et al., 2018; Pillet et al., 2015). Strawberry *FaSCL8* was suggested as involved in fruit ripening through indirect modulation of the flavonoid pathway through MYB10 regulation (Pillet et al., 2015). MYB transcription factors are known to be involved in the regulation of the phenylpropanoid pathway, and several MYB binding sites were identified in the promoter region of the *SlGRAS10* gene. Expression of *SlGRAS10* was also elevated in flower tissues, indicating a role also during flowering. The involvement of GRAS proteins on flower development was reported in lily, where LiSCL was identified as an inducer of the meiosis-associated genes during microsporogenesis of the lily anther (Morohashi et al., 2003).

Expression analysis of *SlGRAS10* in tomato fruits at the Red Ripe stage from different varieties revealed that ‘Valenciano’ fruits, which do not contain any ripening-delaying mutation, presented a lower expression of *SlGRAS10* compared to both MS4 (*rin* mutation) and

'Penjar' (*alc* mutation) varieties (Figure 4.4C). These results suggested that *rin* and *alc* mutations might affect *SlGRAS10* expression. MADS-RIN and NAC-NOR were initially thought to be master regulators of fruit ripening (Casals et al., 2012; Martel et al., 2011; Vrebalov et al., 2002); however, recent studies using CRISPR-Cas9 loss-of-function mutation of *MADS-RIN* and *NAC-NOR* genes revealed a less drastic phenotype than naturaly occurring *rin* and *nor* mutations suggesting a more complex regulation of ripening (reviewed by Wang et al., 2020). Nevertheless, MADS-RIN and NAC-NOR transcription factors might still play a role in ripening and could negatively regulate the expression of *SlGRAS10.* Promoter analysis also revealed several binding sites for ripening-associated transcription factors, including the MADS-box and the NAC families. Nevertheless, these expression results are just preliminary and must be interpreted with caution. Further studies regarding the interaction of SlGRAS10 and other ripening-related proteins will be essential.

CRISPR-Cas9 genome editing technology has been applied to tomato genome manipulation with high rates of success, not only in functional studies but also in quality improvement studies (as reviewed in Chapter 1). In this chapter, CRISPR-Cas9 technology is being applied to target loss-of-function mutation of the *SlGRAS10* gene, and two sgRNAs were selected based on position and on- and off-target scores. The first guide was selected at the beginning of the coding sequence and the second targeting the beginning of the GRAS domain. The action of the Cas9 enzyme at the target sites is expected to create frameshift mutations (indels), leading to loss-of-function alleles. Additionally, constructs for *SlGRAS10* overexpression were assembled under the control of CaMV 35S constitutive promoter and E8 fruit-specific promoter.

The work presented in this chapter is preliminary, and the following analyses are being developed by Flávio Soares in the context of his Ph.D. thesis. The following work comprises the genotyping of the target mutations of the primary transformants and the selection of mutated lines, phenotyping the T1 generation, and the over-expression transformation. It must be taken into account that the SlGRAS10 protein sequence has high similarity with SlGRAS9 and exhibited conserved expression patterns, suggesting genetic redundancy (Huang et al., 2015). Therefore, the development of double mutants will be essential to overcome any functional redundancy that might camouflage the effects of *SlGRAS10* mutation and confirm their putative involvement in the ripening process.

Since tomato is a climacteric fruit, a burst of ethylene is produced at the onset of ripening, which has a central role in the regulation of the ripening process through the regulation of many ripening-related genes (Giovannoni, 2001). Several ethylene response elements (ERE) were

identified in the promoter region of the *SlGRAS10* gene, indicating a regulation of *SlGRAS10* expression by ethylene that could be explored in future studies.

Tomato is one of the most cultivated crops worldwide, and improvement of its quality traits has extreme importance to researchers and producers, including high productivity, tolerance to environmental stress conditions, resistance to pathogens, longer shelf-life, and sensory and health value. Unraveling the involvement of the *SlGRAS10* in the ripening process could be further applied for breeding superior tomato varieties. Moreover, since several GRAS proteins were associated with biotic stress response, *SlGRAS10* might also be involved in defense, and its role might be investigated in the future.

4.5. Material and Methods

4.5.1. Sequence and Phylogenetic analysis

The protein sequence of SlGRAS10 was retrieved from Sol Genomics Network database (https://solgenomics.net/). The properties of deduced amino acid sequence, including the theoretical molecular weight and the isoelectric point, were predicted using ProtParam tool from ExPASy portal (https://web.expasy.org/protparam/). Secondary structure was determined using PSIpred tool (Jones, 1999; http://bioinf.cs.ucl.ac.uk/psipred/).

Sequence comparison with GRAS proteins from Arabidopsis (Araport11 version, https://www.arabidopsis.org/Blast/index.jsp), *Solanum lycopersicum* (ITAG 2.40 version, https://solgenomics.net/tools/blast/), and *Vitis vinifera* (V1 version, http://genomes.cribi.unipd.it/grape/blast/blast.php) was performed using BLASTp algorithm. The seven highest hits from each species, with an e-value lower than 1e-20, were selected, and phylogenetic analysis was conducted in MEGA X software (Kumar et al., 2018). Multiple sequence analysis was performed using MUSCLE (Edgar, 2004). The evolutionary history was inferred by using the Maximum Likelihood method and JTT matrix-based model (Jones et al., 1992), and 1000 bootstrap replicates (Felsenstein, 1985). Initial trees for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model and then selecting the topology with superior log likelihood value. The coding data was translated assuming a Standard genetic code table.

4.5.2. Expression analysis

Expression data of the *SlGRAS10* gene was retrieved from TomExpress database (Zouine et al., 2017; http://tomexpress.toulouse.inra.fr/) for both MicroTom and Heinz 1706 varieties in root, leaf, flower, fruit, and seed.

Additionally, total RNA was extracted from tomato fruits at the Red Ripe stage from three different varieties (MS4, TRVA002 'Valenciano', and TRVA003 'Penjar') and two hybrids (TRVA002 x MS4 and TRVA003 x MS4). Deep frozen tomato powder was kindly provided by José Luis Rambla (Plant Genomics and Biotechnology Lab, IBMCP, Spain). Total RNA was extracted from 200mg of ground tissue using the NucleoSpin® RNA Plant kit (Macherey-Nagel) and treated with the DNA-*free*™ kit DNase treatment (Ambion, Invitrogen), according to the manufacturer's instructions. First-strand cDNA was synthesized from 1 µg of total RNA using PrimeScript™ 1st strand cDNA Synthesis kit (Takara). Real-time PCR reactions were prepared using the SYBR Green Kit (Takara) and performed using the 7500 Fast Real-time PCR System (Applied Biosystems). Cycling conditions were 95°C for 4 min, 30 cycles of 94°C for 30s, 58°C for 30 s and 72°C for 7s, finishing with 72°C for 10min. Expression levels were calculated using the ΔΔCt method and the *Actin 7* gene as the reference gene. Primer efficiency was evaluated using the LinRegPCR software (Ruijter et al., 2009). Primers were designed using the Primer3Plus software (Untergasser et al., 2007) and listed in Table 4.6. Actin 7 primers were kindly provided by Flor Cocaliadis (Plant Genomics and Biotechnology Lab, IBMCP, Spain).

Table 4.4. - List of primers used in the real-time PCR.

Gene	Primer Forward	Primer Reverse
SlGRAS10	GACGAAAGCGTCACAACAGA	TTTGCGACTCAGTCCCTCTT
Actin 7	CCTCAGCACATTCCAGCAG	CCACCAAACTTCTCCATCCC

4.5.3. Promotor analysis

Cis-acting regulatory elements within the 2.5 kb region up-stream the ATG starting codon of the *SlGRAS10* gene were analyzed using PlantCARE software (Lescot et al., 2002; http://bioinformatics.psb.ugent.be/webtools/plantcare/html/). Identification of transcription factor binding sites (TFBSs) in the same region was conducted using Plant Promoter Analysis Navigator (PlantPAN 3.0) software (Chow et al., 2019; http://plantpan.itps.ncku.edu.tw/).

4.5.4. Plant Material

Tomato (*Solanum lycopersicum* L.) cv. Moneymaker seeds were germinated *in vitro* conditions in a long-day growth chamber set to 16h light/8h dark cycle (250 µmol/m^2/s), at 24°C and with a relative humidity of 60-70%. Seeds were sterilized with 50% of bleach, rinsed in sterile water, and placed in germination media containing one-half strength Murashige and Skoog (MS) medium including vitamins and MES buffer, 1.5% (w/v) sucrose, 1% (w/v) Phyto-agar, pH 5.6-5.8. Seeds were kept on dark for three days and then transferred to the light conditions indicated above. All the experiments were conducted at the Plant Genomics and Biotechnology Lab, IBMCP, Valencia, Spain.

4.5.5. Strains and growth conditions

Escherichia coli Top10 was used for gene cloning, and *Agrobacterium tumefaciens* LBA4404 was used for plant transformation experiments. Both strains were grown in LB medium (Bertani, 1951) under agitation (200 rpm) at 37°C and 28°C, respectively. Chloramphenicol (25 µg/mL), kanamycin (50 µg/mL), and spectinomycin (100 µg/mL) were used for *E. coli* selection. Rifampicin (50 µg/mL) and streptomycin (100 µg/mL) were used for *Agrobacterium* selection. 5-Bromo-4-chloro-3-indolyl-*β*-D-galactopyranoside acid (X-GAL, 40 mg/mL) and isopropylthio-***β***-galactoside (IPTG, 0.5 mM) were used on LB agar plates for the white/blue selection of clones.

4.5.6. Cloning Strategy

4.5.6.1. GuideRNA selection and domestication on level 1

Potential Cas9 targets at the *SlGRAS10* gene were identified using the CRISPR tool from Benchling software (https://benchling.com/crispr) and selected based on PAM type (NGG), the first base of the sequence, that must be a G for the U6-26 promoter, *on* and *off*-target scores, and location. Scores are based on algorithms developed by Doench et al. (2016) and Hsu et al. (2013). Two sgRNAs (sgRNA_1 and sgRNA_2) were selected and incorporated in the GoldenBraid scheme through the GB CRISPR domesticator tool (https://gbcloning.upv.es/do/crispr/). Domesticated primers (Table 4.5) were resuspended in water to a final concentration of 1 µM and, equal volumes of forward and reverse primers were mixed and incubated at room temperature for 30 min. The assembly of the gRNAs on level 1 expression cassettes was performed as described in Vazquez-Vilar et al. (2016). Briefly, *BsaI* restriction–ligation reaction was set up in 12 µl with 1 µl of primers mix, 75 ng of GB1001 (U6-26 promoter), 75 ng of GB0645 (scaffold RNA), 75 ng of pDGB3α1 destination vector, 1.2µl

ligase buffer, 1.2µl BSA 10x, 1µl T4 ligase, 1µl *BsaI*. The reactions were set up in a thermocycler for 50 cycles of digestion/ligation reactions (3 min at 37 °C for digestion, 4 min at 16 °C for ligation). One microliter of the reaction was transformed into *E. coli* TOP10 electrocompetent cells. Positive clones were selected by incubation overnight at 37 °C in LB agar plates containing kanamycin (50 µg/mL), IPTG (0.5 mM) and X-gal (40 µg/mL). Plasmid DNA was extracted from white colonies using the E.Z.N.A. Plasmid Mini Kit I (Omega). gRNA constructs were verified by restriction enzyme analysis and later sequencing.

4.5.6.2. SlGRAS10 gene domestication

The DNA sequence corresponding to *SlGRAS10* (Solyc03g025170.1.1) was retrieved from Sol Genomics Network (https://solgenomics.net/) and amplified using specific primers (Table 4.5) from genomic DNA obtained from young leaves of tomato var. Moneymaker. GB domestication primers were obtained using the GB domesticator tool (https://gbcloning.upv.es/do/domestication/), and PCR amplification was performed using the Phusion High-Fidelity DNA polymerase (Thermo Scientific). For domestication, 40 ng of the PCR products were cloned into the pUPD2 with a *BsmBI* restriction–ligation reaction as described previously. The domesticated vector was validated by restriction enzyme (RE) analysis and later sequencing.

Table 4.5 - List of primers used in domestication.

sgRNA	Primer Forward	Primer Reverse
sgRNA_1	ATTGCGAGATTTACGGCGCCGGT	AAACACCGGCGCCGTAAATCTCG
sgRNA_2	ATTGTTCAAGCGCGACCGTAAAG	AAACCTTTACGGTCGCGCTTGAA
SlGRAS10_1	GCGCCGTCTCGCTCGAATGTCGTCAGGTTTCTCCGG	GCGCCGTCTCGGCCTCCCTTGATGAACCATC
SlGRAS10_2	GCGCCGTCTCGAGGCCTAATTTGATGGGGAAG	GCGCCGTCTCGGCGACGAAACCCCTGTAGAA
SlGRAS10_3	GCGCCGTCTCGTCGCTAAATCCGGTTCAAAAC	GCGCCGTCTCGTTGTCTCCGACCACTCGTTG
SlGRAS10_4	GCGCCGTCTCGACAATACAGAATCTGATTAGCCC	GCGCCGTCTCGCTCAAAGCTTAACGCCAAGCTGACGCGA

4.5.6.3. Cloning in α and Ω-level destination vectors

Subsequent *BsaI* or *BsmBI* restriction–ligation reactions were carried out to obtain all the level ≥1 assemblies as described by Sarrion-Perdigones et al. (2013). Level ≥1 constructs were validated by restriction enzyme analysis. Table 4.5 displays all GBparts used in this work.

Table 4.6 – List of GBparts used in GB cloning.

GB Accession	Name
GB1001	pUPD pU6-26 (PROM DPolIII)
GB0645	pUPD psgRNA (SgRNA)
GB0639	pEGB2 alpha2 35s:hCas9:tNos (TU)
GB1103	pEGB2 omega2 SF-35s:hCas9:tNos (Module)
GB1181	pEGB3 omega1R Tnos:NptII:Pnos-SF (Module)
GB0226	pEGB1 alpha1R Tnos:NptII:Pnos (TU)
GB0914	pUPD pPromE8 (PROM+5UTR)
GB0144	pUPD pTE8 (3UTR+TERM)
GB0030	pUPD pP35S (PROM+5UTR)
GB0037	pUPD Tnos (3UTR+TERM)

4.5.7. *Agrobacterium tumefaciens* transformation

The plasmids containing the final multigene modules were purified from *E. coli* and transformed into 50 μL of electrocompetent *Agrobacterium tumefaciens* LB4404 by electroporation at 1440 V, resuspended in 400 μL of SOC medium (Hanahan, 1983) and incubated in a shaker at 28 °C for 2 h. Clones were selected by incubation at 28 °C for two days in LB agar plates containing antibiotics. Positive colonies were grown overnight for two days in 5 mL of LB with selection antibiotics. Plasmid DNA was extracted by QIAprep Spin Miniprep kit (Qiagen), and the assemblies were confirmed by restriction analysis.

4.5.8. Plant Stable Transformation

Agrobacterium tumefaciens mediated stable transformation of cotyledon-explants was performed as described by Ellul et al. (2003) with minor modifications. Briefly, cotyledon explants from approximately 9 days old seedlings were cut transversely into two segments and co-cultivated with *Agrobacterium* inoculum for two days. Explants were then transferred to *callus* induction medium containing MS medium supplemented with vitamins and MES buffer, 1 mg/L IAA and 0.75 mg/L *trans*-zeatin. Generated *calli* were then transferred to shoot induction media supplemented with IAA (0.1 mg/L) and *trans*-zeatin (0.75 mg/L). Developed shoots developed were transferred to shoot elongation media (*trans*-zeatin 0.1 mg/L), and when reached around 2 cm in height were cut off and transferred to rooting media supplemented with 0.2 mg/L IBA to obtain whole plants. Transgenic plants were selected using kanamycin (100 mg/L) as a selection marker.

4.6. References

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Chapter 5

Main Conclusions and Future Perspectives

5.1. Main conclusions and future perspectives

Grapevine is one of the most economically important fruit species worldwide. Therefore, improvement of berry yield and quality, as well as resistance to several diseases, are major concerns in viticulture. Grapevine is susceptible to multiple diseases, such as powdery and downy mildew, gray mold, black rot and trunk diseases. Nowadays, the use of fungicides is part of routine viticulture practices, which might have a negative impact on the environment and in public health, as well as in the cost of grape production. Several resistance *loci* have been identified in resistant species and introgress with *V. vinifera* plants to confer resistance and reduce the impact of disease management. However, most of the hybrids lack the organoleptic properties desired in the wine industry. Moreover, the durability in the field of resistance conferred by a single resistance locus was shown to be low due to the development of resistance-breaking isolates with new avirulence alleles, and loss of resistance has already been described in grapevine (Peressotti et al., 2010). *S*-genes are gaining popularity in the pursuit of resistance and loss of susceptibility is supposed to be broad-spectrum and more durable (Pavan et al., 2010). The application of New Plant Breeding Techniques (NPBT) would be applied to the development of new varieties in a faster and targeted manner than with conventional breeding programs. CRISPR technology is an NPBT that is showing tremendous advantages over the technologies available. This technology is rapidly becoming one of the most used in functional studies, clinical research, and crop improvement due to its ease, low cost, and efficiency. The studies presented in Chapter 1 (Section 1.4) indicate the beginning of a new era in genetic engineering of fruit crops. Still, CRISPR is a very recent technology and further studies are needed to explore its full potential. The most advantageous feature of the CRISPR system over the other NPBTs used until now is the production of cisgenic plants without any trace of transgenic genes for biotechnological purposes. Nevertheless, the classification of these organisms is still on debate. In fact, cisgenic plants obtain with CRISPR-Cas9 technology might not be distinguished from the wild type, and the scientific community does not agree in consider these organisms as transgenic plants. A consensus among the scientific community and political institutions should be achieved in the short term, so this highly promising technology could be implemented in agriculture in the near future.

Understanding the mechanisms underlying the grape berry susceptibility to pathogens and the impact in berry metabolism is essential to improve grapevine characteristics and vinicultural practices and has been the main focus of research in our group. Despite most of the research breakthroughs were related to molecular and biochemical approaches, the final goal is

their applicability to the grape and wine industry. In fact, a microfluidic device was recently developed for the detection of pathogen infections in grapes through the monitorization of the concentration of azelaic acid (Brás et al., 2019). This device was developed based on a previous study in which combined transcriptomic and metabolomic analysis of the grape berry response to *Botrytis cinerea* resulted in the identification of biomarkers of infection, including the azelaic acid, that could be tested for the monitoring of the infection in field conditions (Agudelo-Romero et al., 2015).

Powdery mildew is caused by the biotrophic fungus *Erysiphe necator* and it is one of the most common diseases affecting *Vitis* species. In the work described in Chapter 2, we aim to explore the mechanisms associated with grape berry response to powdery mildew since most of the studies realized so far were focused on the grape leaves. This work was the first to use transcriptomic, metabolomic and hormonal profiling approaches in powdery mildew infected berries, and results revealed that defense mechanisms were activated in 'Carignan' berries, a very susceptible cultivar; however, this activation was not enough to confer resistance. Induction of defense-associated genes was previously observed in leaves from susceptible grapevines (Fekete et al., 2009; Fung et al., 2008; Toth et al., 2016). Nevertheless, some organ-specific responses could be observed in the study presented in Chapter 2, namely related to fatty acid metabolism and isoprenoid biosynthesis. Further studies on the comparison of responses among different plant organs are required to clearly reveal organ-specific defense strategies. The fungal growth stage might also influence the plant response, so it would be important to monitor the fungal development during infection using microscopy approaches.

Some metabolites were also identified as candidate biomarkers of grape infection. One example is the long-chain saturated fatty acids, such as docosanoic and eicosanoic acids, that could be explored as markers for early detection in the field since they were accumulated in grape berries at earlier ripening stages in response to powdery mildew, as well as in fully developed 'Chardonnay' berries infected with powdery mildew (Petrovic et al., 2017). Accumulation of long-chain fatty acids was also reported in berries infected with *B. cinerea* (Agudelo-Romero et al., 2015), suggesting an involvement in response against both biotrophs and necrotrophs. Although several studies have been performed so far, the involvement of long-chain fatty acids in biotic stress is very complex and further investigations are required. One example could be a more complete lipidomic analysis of both berries and leaves during infection, since transcriptomics indicates a different regulation among these tissues. Since long chain fatty acids are one of the main components of the plant cuticular waxes, it would be also

useful to evaluate the fatty acid accumulation in the berry skin and pulp, separately, to confirm if the accumulation is restricted to the berry cuticle.

As a biotrophic fungus, *E. necator* relies on the host nutrients to proceed with its life cycle. Several genes related to sugar metabolism and mobilization were up-regulated in response to powdery mildew. Induction of genes coding sugar transporters, as well as the cell wall invertase, has been reported in response to several pathogens (Chen et al., 2010; Hayes et al., 2010). Nevertheless, in our study glucose and fructose levels were not significantly affected. It could be hypothesized that the host cells were able to maintain the basal sugar levels, despite fungal consumption. This balance between fungal uptake and host accumulation could be related to the up-regulation of sucrose synthase coding genes, which converts sucrose into fructose and glucose. Although studies in wheat and pea reported that glucose is the principal sugar transferred to the powdery mildew mycelium (Clark and Hall, 1998; Sutton et al., 2007, 1999), it is also possible that the grapevine fungus might be uptaking other minor sugars as well, such as galactose. Several transcripts coding sugar transporters, including sucrose, glucose, and UDP-galactose transporters, were detected in the fungus transcriptome associated with the biotrophic interaction. More focused work on the sugar mobilization during infection would be essential to understand how the fungus influences the sugar homeostasis within host cells, through the uncovering of which sugars are translocated from the berries to the *E. necator* mycelium using radiolabeled compounds, and if it changes during ripening. Another interesting approach would be the study of source-sink relationships through measuring the photosynthetic rate, sugar accumulation and expression of sugar transporters coding genes in both berry clusters and surrounding leaves from infected and non-infected plants in multiple stages of berry development, since an overall down-regulation of photosynthesis genes was reported in infected 'Cabernet Sauvignon' leaves (Fung et al., 2008) and in response to *véraison* in berries infected with powdery mildew, as supported by the enrichment analysis.

Phenylpropanoid pathway was also activated in response to powdery mildew and could be a good target for grapevine improvement, namely the stilbenoid pathway. Accumulation of stilbenoids has been correlated with higher tolerance to powdery mildew infection (Belhadj et al., 2006; Schnee et al., 2008). Moreover, phenolics are important contributors to the organoleptic properties of the wine and have a positive impact on human health, due to their antioxidant properties.

This work was also the first to analyze the hormonal profile of infected grape berries, reinforcing the central role of salicylic acid in response to biotrophic pathogens, particularly in response to powdery mildew. Furthermore, jasmonates, which are classically associated with

plant signaling of necrotrophs' infection, were modulated in response to powdery mildew. The action of jasmonates in the context of biotrophs invasion is an interesting topic for further studies. It would be interesting to compare the hormonal profile of berries from susceptible plants with berries from resistant plants. Higher basal levels of salicylic acid would be expected for resistant berries as observed in leaves (Appendix - Figure 1; Fung et al., 2008). Furthermore, a reduction of the OH-JA-Ile levels was observed in leaves after 6h of infection with powdery mildew in both resistant and susceptible plants (Appendix – Figure 1), contrary to what tends to happen in infected berries, reinforcing the need to explore organ-specific responses.

Transcription factors are key regulators of the plant processes, regulating the expression of several genes. Several genes coding transcription factors were modulated in response to powdery mildew, more specifically genes coding WRKY and MYB transcription factors, as previously observed in leaves (Fung et al., 2008; Toth et al., 2016). Some *LATERAL ORGAN BOUNDARIES domain* (*LBD*) genes were also responsive to powdery mildew and/ or modulated at *véraison*. LBD transcription factors have crucial roles in plant organ development and specific *LBD* genes have been associated with disease susceptibility, as reviewed in Chapter 1. In Chapter 3, a genome-wide analysis was performed to identify and map the *LBD* genes in the grapevine genome. Fifty *LBD* genes were identified in sixteen of the nineteen grapevine chromosomes. Expression analysis suggested the role of specific genes in fruit ripening as well as in response to stress conditions. *LBD* genes might be good targets for grape quality improvement and increased stress resilience. The study of the involvement of *LBD* genes in powdery mildew susceptibility, namely *VviLBDIa1*, *VviLBDIf13*, and *VvLBDIIb1*, could provide candidates for improved resistance through the loss of susceptibility. Recent studies showed that targeted gene mutation of the citrus *LOB1* gene was shown to induce resistance to citrus canker (Jia et al., 2017; Peng et al., 2017).

In the present work, some *GRAS* genes were also found to be modulated in response to powdery mildew and at the onset of ripening. GRAS transcription factor family has been associated with several plant development processes and signaling (Bolle, 2016). Certain *GRAS* genes were shown to be modulated in grape berries in response to *Botrytis cinerea* infection (Agudelo-Romero et al., 2015). Several *GRAS* genes were also reported to be involved in the onset of ripening (Grimplet et al., 2016). Grapevine *VviPAT6* and tomato *SlGRAS10* orthologs, previously identified as putative regulators of fruit ripening in both non-climacteric and climacteric plants (Grimplet et al., 2016), were selected for functional characterization. In Chapter 4, *slgras10* mutants were developed using CRISPR-Cas9 technology. Further analysis of the effect of the mutation on the tomato ripening is being conducted in our group. As referred

in Chapter 4, SlGRAS10 has high similarity with SlGRAS9; therefore, double mutants using two gRNAs targeting each gene are being created to overcome any functional redundancy. In parallel, *VviPAT6* is being characterized in order to study its role in grape berry ripening and to verify if both orthologs have a similar function. Proving the role of *SlGRAS10* in tomato ripening, it could be a good target for improvement of quality traits, such as increasing shelf life. The involvement of *GRAS* genes in stress responses might also be explored in the future.

Taking all together, the results obtained in this work clarified the effect of powdery mildew in berry metabolism of susceptible grapevines at early ripening stages. It also suggested an involvement of *LBD* and *GRAS* genes in grape ripening and in response mechanisms associated with powdery mildew. These findings might be deeply explored and ultimately applied in the improvement of grapevine yield, resistance to pathogens and berry quality through the use of NPBT, such as CRISPR technologies, as well as in the adjustment of agricultural practices and the development of devices for early disease detection.

5.2. References

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Appendix

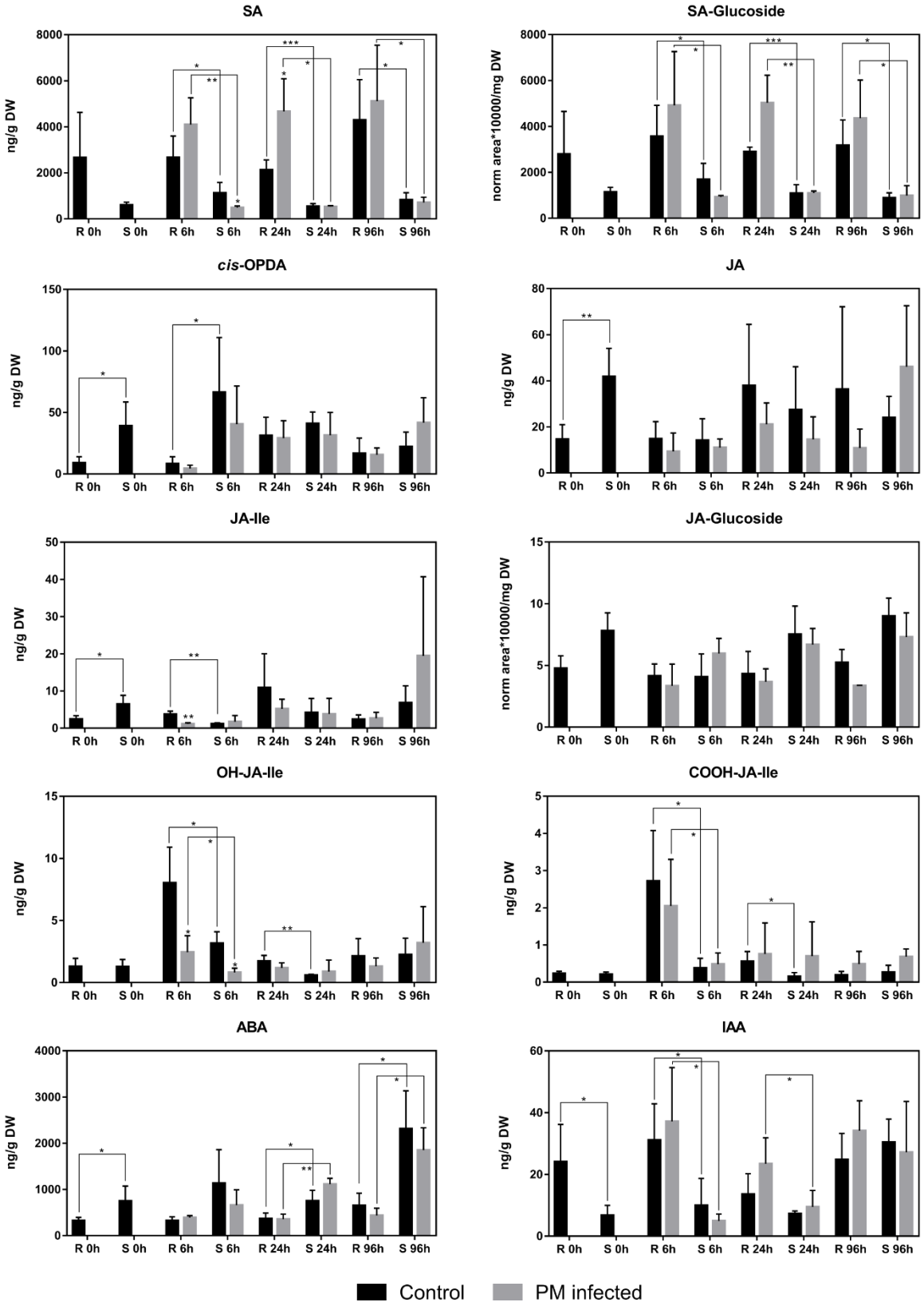

Figure 1 - Hormonal profiling of powdery mildew (PM) infected and control leaves from susceptible (S), *Vitis vinifera* cv. Aragonês, and resistant (R) plants, *V. riparia* X *V. rupestris* 101-14 Millardet et de Grasset. Grapevine plants were infected by contact with naturally infected leaves and collected at four time points: 0 hpi (hours post infection), 6 hpi, 24 hpi and 96 hpi. Bars and whiskers represent averages and standard deviation (SD). Asterisks

indicate statistical significance on pairwise comparisons (*Student's t-test:* * p-value ≤ 0.05; ** p-value ≤ 0.01; *** p-value≤0.001). SA, salicylic acid; SA-Glucoside, salicylic acid-*β*-D-glucoside; *cis*-OPDA, 12-oxo-phytodienoic acid; JA, jasmonic acid; JA-Glucoside, 12-*O*-glucoside-jasmonic acid; JA-Ile, conjugate jasmonoyl isoleucine; OH-JA-Ile, hydroxyjasmonoyl-isoleucine; COOH-JA-Ile, dicarboxyjasmonoyl-isoleucine; ABA, abscisic acid; IAA, indole acetic acid.