Epigenetic marks in Grapevine – *Plasmopara viticola* interaction:
The role of DNA Methyltransferases

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Resumo alargado

Vitis Vinifera L. é conhecida por ser uma das plantas mais cultivadas a nível mundial, com um grande impacto económico e agrícola. Com cerca de 7,5 milhões de hectares de produção a nível global, o produto final pode ser utilizado como uvas secas (passas) ou uvas de mesa, assim como para fabrico de vinho. A produção de vinho é uma prática excecionalmente relevante em vários países e como tal, mais de metade das uvas produzidas são utilizadas para esse efeito. Portugal é um dos maiores produtores de vinho a nível europeu, ocupando a quinta posição em 2019. Apresentando mais de 250 variedades nacionais, a cultura vinícola portuguesa é muito rica, o que torna a indústria no nosso país uma das mais conceituadas e apreciadas no mundo. No entanto, tanto em Portugal como noutros países vitivinícolas, a videira é afetada por um número considerável de agentes patogénicos que, devido a danos e perdas causados na qualidade e rendimento da vinha, têm um impacto negativo na produção de vinho.

A doença do míldio é uma das doenças mais conhecidas e devastadoras de *Vitis vinifera*. Esta doença é causada pelo oomicete biotrófico obrigatório *Plasmopara viticola* (Berk. et Curt.) Berl. et de Toni, nativo da América do Norte. Este patógeno foi introduzido na Europa acidentalmente no século XIX quando espécies nativas americanas de *Vitis* foram usadas como porta enxertos para prevenir a infeção por filoxera. Uma vez que as espécies europeias evoluíram sem entrar em contacto com o *P. viticola*, são altamente suscetíveis a este agente patogénico. Após ser introduzido em França, este patógeno alastrou-se rapidamente pelas regiões vinícolas europeias, levando a graves perdas de produção, graças à sua capacidade de devastar culturas inteiras de vinha.

Dependendo inteiramente do seu hospedeiro para completar o seu ciclo de vida, o *P. viticola* inicia o processo de infeção quando as condições ambientais são favoráveis, isto é, quando a temperatura se encontra entre os 21 e os 25ºC e a humidade por volta dos 80%. Neste processo, o esporângio maduro liberta zoósporos que são capazes de infetar tecidos vegetais. Estas estruturas germinam na superfície abaxial de folhas e penetram através da cavidade estomática, criando uma vesícula responsável por dar origem às hifas primárias e ao micélio.
Os sintomas de infeção por *P. viticola* nas folhas de videira ocorrem, primeiramente, através da descoloração das lesões (lesão amarelada ou manchas de óleo). Posteriormente os esporangióforos emergem através da cavidade estomática, libertando esporângios para os tecidos e plantas circundantes.

Atualmente a estratégia mais utilizada para controlar o míldio da videira está dependente da aplicação preventiva de fungicidas durante a época de cultivo. No entanto, foram manifestadas preocupações relativamente aos riscos que esta poderia causar, tanto para a saúde humana como para o ambiente. Assim sendo, foram estabelecidas diretrizes por parte da União Europeia para a redução do uso de pesticidas e fungicidas na viticultura. Consequentemente, nos últimos anos, os produtores e investigadores têm tentado encontrar soluções alternativas sustentáveis para controlar esta doença.

A interação entre a videira e este agente patogénico foi amplamente estudada de forma a melhor compreender o processo de infeção, o complexo mecanismo de defesa da planta e a identificar potenciais alvos para estratégias de controlo alternativas ao uso de pesticidas. As abordagens ao nível da transcritómica, metabolómica e proteómica demonstraram que o *P. viticola* tem a capacidade de induzir uma forte e rápida reprogramação ao nível dos transcritos e proteínas, bem como do metabolismo primário e secundário. Alguns estudos demonstraram também que tanto em genótipos de videira suscetíveis como em tolerantes existe uma ativação de mecanismos de defesa. No entanto, na interação incompatível, a planta tem a capacidade de detetar a presença do agente patogénico e ativar a cascata de sinalização mais rapidamente, permitindo assim o estabelecimento de mecanismos de defesa mais eficazes. Em genótipos suscetíveis, apesar de haver uma tentativa de ativação mecanismos de defesa, o patógeno é capaz de ultrapassar as defesas da planta e estabelecer o seu ciclo de vida. Recentemente foi demonstrado que a metilação do DNA também tem um papel muito importante nas interações entre planta e patógeno, nomeadamente através da modulação dos níveis de expressão genética. Vários estudos demonstraram que a hipometilação global do genoma é uma característica comum em interações incompatíveis, possivelmente através da modulação da transcrição. Assim sendo, a procura e aquisição de mais conhecimentos neste campo de investigação que se encontra em rápido crescimento, a epigenética, poderá ser a chave para melhor compreender a imunidade de *V. vinifera* ao *P. viticola*. A metilação do DNA é um exemplo de uma marca epigenética que pode ser responsável por causar reprogramação do transcritoma durante as interações entre planta e patógeno. Contudo, a principal função da metilação do DNA no estabelecimento de interações compatíveis e incompatíveis permanece maioritariamente inexplorada.

O principal objetivo deste trabalho passa por identificar e perceber qual o papel das DNA metiltransferases de *Vitis vinifera* no estabelecimento de interações compatíveis e incompatíveis entre a videira e o patógeno *P. viticola*.

As DNA metiltransferases reconhecem uma sequência específica de DNA para a qual transferem um grupo metil para o carbono número cinco do anel de pirimidina dos resíduos de citosina. A metilação da citosina é essencial em algumas funções celulares das plantas e pode ocorrer de forma simetrica (CG ou CHG) ou assimétrica (CHH), onde o H pode corresponder a qualquer nucleótido que não seja guanina.

De forma a conseguir alcançar o primeiro objetivo traçado para este trabalho, foi efetuada uma pesquisa em bases de dados e comparação com outros organismos modelo onde as DNA metiltransferases já tinham sido previamente descritas. Desta forma, foi possível detetar os genes de DNA metiltransferases em *Vitis vinifera*. Foram identificados 9 genes que codificam para 17 possíveis proteínas (resultantes de eventos de splicing alternativo), sendo que estes genes se encontram distribuídos em oito dos dezanove cromossomas de *Vitis vinifera*. Foi também demonstrado neste estudo que alguns dos genes de metiltransferases em videira estão localizados perto de *loci* de resistência ao *P. viticola*.

As metiltransferases identificadas foram agrupadas em quatro grupos de acordo com a semelhança entre a sequência das proteínas, resultantes de uma análise filogenética, nomeadamente *methyltransferase* (MET), *chromomethylases* (CMT), *domains rearranged methyltransferase* (DRM), e

DNA methyltransferase homolog (DNMT). Foi também prevista a presença de quatro domínios diferentes nas DNA metiltransferases da videira, nomeadamente DNMT1-RFD, BAH, Chromo e DCM. Uma análise a nível da localização subcelular revelou que, embora a maioria das metiltransferases de videira estejam localizadas no núcleo, existem algumas presentes no cloroplasto e vacúolo.

O segundo objetivo deste projeto consistiu em determinar e correlacionar os padrões globais de metilação de DNA com a expressão dos genes de DNA metiltransferases em duas cultivares, suscetível e tolerante, de *Vitis vinifera*, tolerante e suscetível, após a inoculação com o *P. viticola*.
Os resultados obtidos sugerem um papel relevante da hipometilação do DNA na cultivar tolerante ao patógeno, com uma maior capacidade de resistir ao ataque do *P. viticola*. Para além disso, dois dos genes estudados demonstraram também poder estar envolvidos na modulação epigenética associada ao estabelecimento da interação incompatível.

Futuramente, novos estudos serão realizados no âmbito de melhor compreender os padrões de metilação dos genes da família de metiltransferases nas folhas de videira.

Palavras-chave:
Vitis vinifera; *Plasmopara viticola*; Epigenética; Metilação; DNA metiltransferases

Resumo

Vitis vinifera L. é conhecida mundialmente por ser uma das plantas mais cultivadas, com cerca de 7,5 milhões de hectares de produção. De todas as utilidades possíveis, a produção de vinho é a prática mais comum e é excecionalmente relevante em vários países. No entanto, a videira encontra-se sujeita a um número considerável de patógenos que influenciam de forma negativa a qualidade e rendimento da vinha, afetando assim a sua produção. A doença do míldio, causada pelo oomycete biotrófico *Plasmopara viticola* (Berk. et Curt.) Berl. et de Toni, foi introduzido na Europa acidentalmente no século XIX. Após a sua dispersão neste continente, a presença deste patógeno levou a graves perdas de produção. Atualmente, as estratégias usadas para lidar com esta doença dependem do uso preventivo de compostos fitoquímicos em cada época de cultivo, representando elevados custos ambientais, económicos e sanitários. Assim sendo, é necessária a caracterização da resistência de algumas espécies de *Vitis* e genótipos de *V. vinifera* ao *Plasmopara viticola* de forma a definir medidas sustentáveis de controlo a esta doença.

As modificações epigenéticas são importantes na indução da diversidade fenotípica, incluindo em respostas de resistência das plantas, através do controlo dos níveis de expressão genética relacionados com a defesa. Neste trabalho foi possível identificar e caracterizar nove genes de DNA metiltransferases presentes em *Vitis vinifera*. Estes genes encontram-se distribuídos em oito dos dezanove cromossomas conhecidos de *Vitis*, estando alguns localizados perto de genes que estão associados à resistência de *Vitis vinifera* contra o *P. viticola*. Foi possível agrupar as metiltransferases identificadas em quatro grupos já conhecidos em plantas, nomeadamente *methyltransferase* (MET), *chromomethylases* (CMT), *domains rearranged methyltransferase* (DRM), e *DNA methyltransferase homolog* (DNMT). Foram determinados e correlacionados os padrões globais de metilação de DNA com a expressão dos genes de DNA metiltransferases em duas cultivares distintas de *Vitis vinifera*, tolerante e suscetível, após inoculação com o *Plasmopara viticola*. Os níveis de metilação do DNA genómico observado para ambas as cultivares foi distinto. A expressão das metiltransferases estudadas apresentou alguns indícios de presença na regulação da metilação durante a interação entre a videira e o míldio. Os resultados obtidos sugeriram haver uma relação entre a hipometilação e a cultivar tolerante ao patógeno. Deste modo, dois genes demonstraram poder estar envolvidos na modulação epigenética associada ao estabelecimento da interação incompatível.

Novos estudos terão que ser realizados no âmbito de melhor compreender os padrões de metilação dos genes da família de metiltransferases nas folhas de videira.

Palavras-chave:

Vitis vinifera; *Plasmopara viticola*; Epigenética; Metilação; DNA metiltransferases

Abstract

Vitis vinifera L. is known for being one of the most cultivated crops worldwide, with about 7.5 million hectares of production. Of all the possible uses, wine production is the most common and exceptionally relevant practice in several countries. However, the cultivated grapevine, *Vitis vinifera*, is susceptible to a considerable number of pathogens, such as downy mildew, which negatively influences the quality and yield of grapevine, thus affecting wine production. Downy mildew disease, caused by the biotrophic oomycete *Plasmopara viticola* (Berk. et Curt.) Berl. et de Toni, was accidentally introduced in Europe in the 19th century. After spreading throughout the European wine regions, the presence of this pathogen led to severe production losses. Currently, the preventive use of phytochemical compounds on each growing season is the strategy selected to deal with this disease, leading to high environmental, economic and health costs. Therefore, characterization of the resistance of some *Vitis* species and *V. vinifera* genotypes to *Plasmopara viticola* is necessary in order to define sustainable control measures for this disease.

Epigenetic modifications are important in inducing phenotypic diversity, including in plant resistance responses, by controlling defense-related gene expression levels. In this work, it was possible to identify and characterize nine DNA methyltransferases genes present in *Vitis vinifera*. These genes are distributed in eight of the nineteen known chromosomes of *Vitis*, and some are located near genes that are associated with *Vitis vinifera* resistance against *P. viticola*. It was possible to classify the identified methyltransferases into four groups already known in plants, namely methyltransferase (MET), chromomethylases (CMT), domains rearranged methyltransferase (DRM), and DNA methyltransferase homolog (DNMT). Global DNA methylation patterns were determined and correlated with the expression of DNA methyltransferases genes in two distinct cultivars of *Vitis vinifera*, tolerant and susceptible, after inoculation with *P. viticola*. The levels of genomic DNA methylation observed for both cultivars were distinct. The expression of the identified methyltransferases showed some evidence of presence in the regulation of methylation during the interaction between grapevine and downy mildew. The results obtained suggested that there was a relationship between hypomethylation and the cultivar tolerant to the pathogen. Thus, two genes were shown to be involved in the epigenetic modulation associated with the establishment of the incompatible interaction.

Further studies will have to be carried out in the framework of better understanding the methylation patterns of DNA methyltransferases genes in grapevine leaves.

Keywords:

Vitis vinifera; *Plasmopara viticola*; Epigenetics; Methylation; DNA methyltransferases

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Abbreviations

5-mC	5-Methylcytosine
AdoMet	S-adenosyl-L-methionine
ATR	Atrazine
AVR	Avirulence factor
BABA	β-aminobutyric acid
C5-MTases	Cytosine-5 DNA methyltransferases
cDNA	Complementary DNA
CMT	Chromomethylases
DM	Downy Mildew
DMPs	Differentially methylated promoters
DMRs	Differentially methylated regions
DNMT	DNA methyltransferase homolog
dpi	Days post-inoculation
DRM	Domains rearranged methyltransferase
EF1α	Elongation factor 1-alpha
ET	Ethylene
ETI	Effector-triggered immunity
ETS	Effector-triggered susceptibility
gbM	Gene body methylated
hpi	Hours post-inoculation
HR	Hypersensitive response
JA	Jasmonic acid
m6A	N6-methyladenine
MAPKs	Mitogen-activated protein kinases
MET	Methyltransferase
Mhl	Million hectoliters
miRNA	MicroRNA
MTases	Methyltransferases
Mw	Molecular weight
NBS-R	Nucleotide-binding site-resistant
PAMP	Pathogen associated molecular patterns
PCD	Programmed cell death
pI	Isoelectric point
PRRs	Pattern recognition receptors
PTI	PAMP triggered immunity
qPCR	Quantitative real-time polymerase chain reaction
QTL	Quantitative Trait Loci

R proteins	Resistance proteins
RdDM	RNA-directed DNA methylation
ROS	Reactive oxygen species
RPV	“Resistance to *Plasmopara viticola*”
SA	Salicylic acid
SAND	*SAND family protein*
SAR	Systemic acquired resistance
siRNA	Small interfering RNA
TEs	Transposable elements
TGS	Transcriptional gene silencing
TSS	Transcriptional start site
TTS	Transcriptional termination sites
UM	Unmethylated
WGBS	Whole-genome bisulfite sequencing

1. Introduction

1.1. The economic and historical relevance of *Vitis vinifera* L.

Grapevine (*Vitis vinifera* L.), belonging to the Vitaceae family, is one of the crops with the highest economic and agricultural impact worldwide (Terral *et al.* 2010; Buonassisi *et al.* 2017). Grapevine history accompanied human development, with the first reports of *Vitis* species presence in the European continent dating from 50 million years ago. Grapevine domestication started in Near East Europe (a region corresponding to the modern Middle East) around 6000-8000 years ago, from its wild relative *Vitis vinifera* L. ssp. *sylvestris.* Due to ancient civilizations' trade routes, it spread across Europe, Asia, and North Africa (Zdunić *et al.*, 2013). The Roman Empire was also responsible for introducing new cultivars in the Lusitanian viticulture (Veloso *et al.*, 2010). The domestication of grapevine selected essential traits such as berry size and production yield. However, it led to a reduction in genetic diversity and loss of resistance traits such as pathogen resistance (Terral *et al.*, 2010).

Nowadays, viticulture is a worldwide practice, with a global area of around 7.4 million ha in 2019 and a total grape production of 77.8 million tons, to be used as dried and table grape as well as in wine production (OIV 2020). More than half of the produced grapes are destined for wine production since this industry is exceptionally relevant in several countries. In 2019, the wine production exceeded 260 million hectoliters (Mhl) in the world, with the EU production representing 60% of global production and Portugal standing in the fifth position within the European countries (FAOSTAT 2019; OIV 2020)

Despite the current circumstances where geopolitical tensions, climate change, and covid-19 pandemic are generating a lot of instability and insecurity in the global wine market, 2020 world wine production seems to be in line with that observed in 2019. This is also true for the Portuguese wine production, that is comparable to that of 2019 and its last five-year average of 6.5 Mhl (OIV 2020).

1.2. Grapevine downy mildew – epidemiology

Grapevine is affected by a considerable number of pathogens that present a high impact on wine production globally due to berry damage and losses in both quality and yield (Santos & Figueiredo, 2021).

Gray mold, powdery mildew and downy mildew caused by *Botrytis cinerea* Pers. Fr., *Erysiphe necator* (Schwein.) and *Plasmopara viticola* (Berk. And Curt.) Berl. & de Toni., respectively, are known to be the most important diseases of *Vitis vinifera*. Of those, downy mildew is undoubtedly one of the most devastating (Williamson *et al.*, 2007; Milli *et al.*, 2012; Qiu *et al*., 2015; Armijo *et al.*, 2016; Santos & Figueiredo, 2021).

Plasmopara viticola, first observed and collected in 1834 in the USA's North East, is an obligatory biotrophic oomycete that relies entirely on its host to complete the life cycle (Armijo *et al.*, 2016; Buonassisi *et al.*, 2017). This pathogen is native to North America and was accidentally introduced in

Europe when native American Vitis species were used as rootstocks to prevent infection from *Phylloxera*. As the European Vitis species evolved without contact with *P. viticola*, *V. vinifera* plants are highly susceptible to this pathogen. Thus, after its introduction in France in 1878, it spread rapidly into major European grape production regions causing severe production losses (Yin *et al*., 2017; Taylor *et al.,* 2019).

The infection caused by *P. viticola* occurs when environmental conditions are favorable, namely high humidity and mild temperatures (Jürges *et al.*, 2009). With temperatures above 10ºC and rain precipitation, sporangia are produced after oospores germination or by mycelium present in fallen leaves and berries or dormant twigs, infected during previous seasons. In the presence of rain or wind, mature sporangia are released and disperse zoospores capable of infecting plant tissues (Burruano 2000; Armijo *et al.* 2016; Buonassisi *et al.* 2017; Brilli *et al.* 2018; Toffolatti *et al.* 2018). Motile zoospores can place themselves close to stomata on leaves' abaxial surface (Armijo *et al*., 2016). After germination, zoospores penetrate through the stomatal cavity, where a vesicle will be created, in order to develop into a primary hyphae and intercellular mycelium (Armijo *et al*., 2016). Commonly, in a susceptible cultivar, the first visible symptoms appear on the abaxial surface of the leaf 5-7 days after leaf infection, characterized by green-yellow lesions that are also called oil spots (Burruano 2000; Armijo *et al.* 2016; Buonassisi *et al.* 2017).

Secondary infections are prompted by sporangiophores that emerge through the stomata. Detachment and dispersion of sporangia to new host tissues initiates a new infection cycle. *Plasmopara viticola* is diploid in both sexual (primary infection) and asexual stages (secondary infection) and, under appropriate conditions, secondary disease cycles can take place. Through the fertilization of oogonia by antheridia, this asexual stage happens within the infected host tissue. An oospore is considered the pathogen's survival stage, representing the primary inoculum for the next season and a source of genetic variation. Several clonal cycles may occur in one season, depending on environmental conditions (Burruano 2000; Buonassisi *et al.* 2017).

Downy mildew disease control relies on preventive fungicide applications during the crop season, depending on the meteorological conditions (Gindro *et al.,* 2006; Buonassisi *et al.,* 2017; EIP-AGRI 2019). Consequently, concerns have been raised regarding the risks for human health and adverse environmental effects. It is known that less than 0.1% of fungicides applied on crops actually act on the pathogen (Buonassisi *et al*., 2017). The remaining 99.9% accumulate in soils, sifting into the ground or shallow water, and might be toxic to some microorganisms as well as aquatic animals and humans (Wilson & Tisdell, 2001; Buonassisi *et al.*, 2017). Given that, there is a European Union demand for reducing the use of pesticides and fungicides in viticulture (Directive 2009/128/EC). Hence, in the past few years, producers and researchers have tried to find sustainable alternative solutions to control diseases.

1.3. Grapevine resistance to *Plasmopara viticola* - current knowledge

Plants recognize an invading agent by activating defense mechanisms to restrain pathogen growth. On the other hand, pathogens have the power to manipulate the plant, so it harbors the best environment for its own growth and development by avoiding the plant response. Thus, the interaction between plants and pathogens can be seen as a process that implicates mutual communication between these two organisms (Méndez & Romero 2017). It can be classified as compatible interaction (when the infection is successful, leading to the disease) or incompatible interaction (where the plant defense mechanisms act successfully, impairing pathogen growth) (Ponzio *et al.*, 2016).

The molecular mechanisms associated with pathogen recognition and disease resistance in plants have started to be unraveled in the past years (Burgh & Joosten 2019).
Jones and Dangl developed a model describing the evolutionary battle between plants and pathogens, known as the 'zig-zag model' (see Figure 1) (Jones & Dangl 2006). This model proposes the existence of two layers of defense in the plant immune system that the pathogen tries to avoid by diversifying its own effectors and developing new ones, while the plant evolves new receptors so it can detect a broader range of pathogen effectors (Buonassisi *et al.* 2017).
PTI (PAMP (pathogen-associated molecular patterns) triggered immunity) is known as the first defense level, providing plants with the capacity to respond rapidly and efficiently to a wide variety of pathogens. This first layer of defense is activated through the recognition of conserved pathogen molecular patterns, such as cell wall components, short peptides and lipopolysaccharides, by specific pattern recognition receptors (PRRs) on the plasma membrane of plant cells (Ding & Wang 2015; Buonassisi *et al.* 2017; Gouveia *et al.* 2017). During PTI, early defense responses such as reactive oxygen species (ROS), calcium flux, plant cell wall modification and the activation of a cascade of mitogen-activated protein kinases (MAPKs) are triggered (Ding & Wang 2015). This first response is usually enough to defeat the invading pathogen. However, some pathogens have developed the ability to overcome PTI through the secretion of effectors, establishing the effector-triggered susceptibility (ETS). Pathogen effectors can be responsible for, as an example, stimulating nutrient leakage, thus suppressing the plant immune response as well as manipulating the physiology of the host cell *(*Ding & Wang 2015; Buonassisi *et al.* 2017). To overcome ETS, plants have developed a second level of resistance associated with effector recognition by specific intracellular receptors or sensors, known as resistance proteins (R). When an R protein recognizes pathogen effectors, effector-triggered immunity (ETI) is activated. ETI is the second defense level regularly associated with the establishment of the hypersensitive response (HR) with fast cell death at the point of infection, which may restrict pathogen growth. ETI is often related to ROS and salicylic acid (SA) production, expression of defense-related genes and the establishment of the HR (Ding & Wang 2015; Merz *et al.* 2015; Kelloniemi *et al.* 2015; Armijo *et al.* 2016; Buonassisi *et al.* 2017; Gouveia *et al.* 2017).

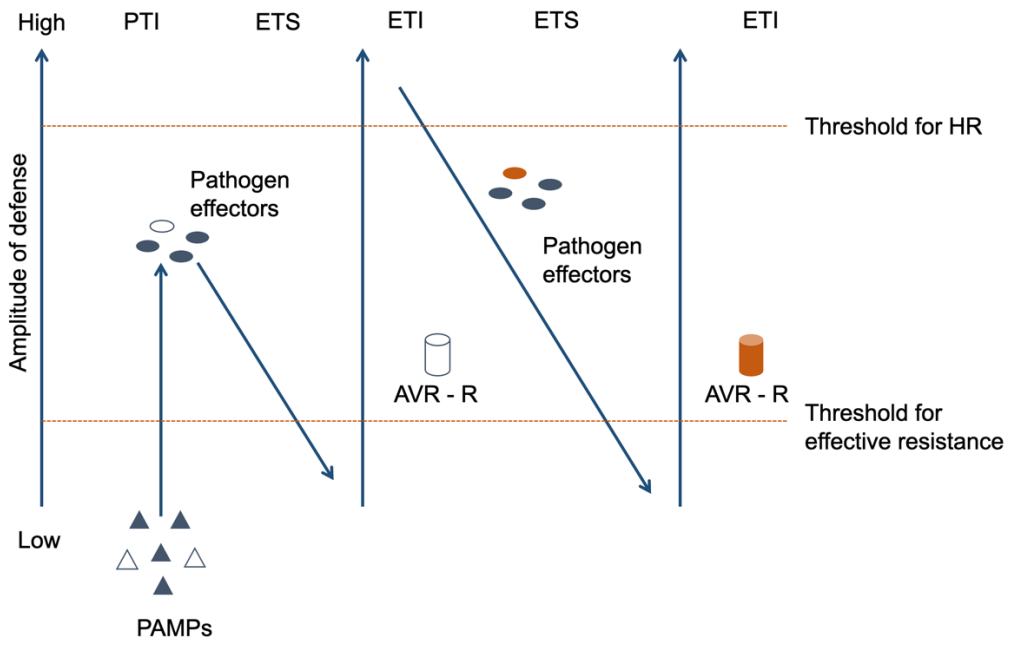


Figure 1 – The "zig-zag model" described by Jones and Dangl in 2006. PTI – is the first defense level, defined as PAMP (pathogen-associated molecular patterns) triggered immunity; When this first defense mechanism is not enough for defeat the invading pathogen, some plants have the ability to overcome PTI through the secretion of effectors, establishing the ETS, known as effector-triggered susceptibility; To overcome ETS, plants developed the second defense mechanism, ETI – effector-triggered immunity, associated with the establishment of the hypersensitive response (HR). Adapted from (Jones & Dangl 2006)

Apart from PTI and ETI responses after the initial local infection, plant tissues that were not infected usually develop systemic acquired resistance (SAR), which provides resistance in distal plant tissues against subsequent pathogen challenges (Ding & Wang 2015).

In grapevine-*P. viticola* interaction, the knowledge of early host responses to pathogen attack, and the complex grapevine defense mechanism have increased tremendously over the last decade. This knowledge has been reviewed recently by Buonassisi and colleagues (Buonassisi *et al.*, 2017). Downy mildew infection is often associated with a considerable plant transcriptional reprogramming that accounts for intense and rapid activation of stress and defense-related genes and thus of defense and secondary metabolic processes (Figueiredo *et al.*, 2008, 2012; Ali *et al.*, 2012; Latouche *et al.*, 2013; Yu *et al.*, 2016; Buonassisi *et al.*, 2017; Negrel *et al.*, 2018). Plant resistant genotypes response involve the specific modulation of genes encoding components of signal transduction cascades, markers related to HR, genes linked to stilbene and defense hormone biosynthesis, and activation of photosynthetic apparatus protection (Polesani *et al.*, 2008; Ali *et al.*, 2012; Figueiredo *et al.*, 2012; Liu *et al.*, 2014; Yu *et al.*, 2016; Buonassisi *et al.*, 2017; Laureano *et al.*, 2018). For the establishment of incompatible interactions, the accumulation of lipid-associated signaling proteins, proteases, cell death-related proteins is also crucial (Figueiredo *et al.*, 2017). It was recently highlighted the co-occurrence of proteins associated with both ETS and ETI as soon as 6 hours post inoculation with the pathogen, revealing the high plasticity of tolerant genotypes (Santos *et al.,* 2020). Moreover, genotype-phenotype association approaches have been made, involving the establishment of quantitative trait loci (QTL) analysis and

determination of resistance loci to *Plasmopara viticola* (RPVs) (Buonassisi *et al.*, 2017; Yadav *et al.*, 2018).

1.4. Epigenetics

In 1942, the concept of "epigenetics" was firstly introduced by Conard Waddington to explain the process through which, during organism development, genotypes generate phenotypes (Law & Jacobsen 2010; Gómez-Díaz *et al.* 2012; Bej & Basak 2017). Epigenetics was later re-defined as "*the study of changes in gene function that are mitotically and/or meiotically heritable and that do not entail change in DNA sequence*" (Wu & Morris 2001; Seffer *et al.* 2013).

The epigenetics processes include DNA methylation, histone modifications (acetylation, methylation, phosphorylation, ubiquitination, sumoylation), and chromatin remodeling (Gómez-Díaz *et al.* 2012; Bej & Basak 2017). Small RNA's (sRNA), like microRNA (miRNA), and small interfering RNA (siRNA), recently became the extended part of the epigenetic regulation, being considered the bioregulators of plant stress response. Nowadays, DNA methylation, histone modifications, and RNA-based mechanisms have been considered "the three pillars of epigenetics" as they correlate with each other (Bej & Basak 2017; Sudan *et al.* 2018).

Genetics, cancer genetics, aging, stem cell research, evolution, synthetic biology, conservation of species, plant biotechnology, and agriculture are some of the biological fields that epigenetics have been widely applied to in the last decade (Bej & Basak 2017). However, there is still very little information about the dimension and significance of epigenetic variations in host-pathogen interactions. These interactions are among the most plastic and dynamic systems in nature (Gómez-Díaz *et al.*, 2012).

Developmental and environmental signals can be responsible for inducing epigenetic modifications in the genome, and therefore, one single genome in a plant cell brings on multiple epigenomes (Chinnusamy & Zhu, 2009). Chromatin structure is responsible for affecting the genome expression through processes often associated with epigenetic regulation, namely histone variants, histone post-translational modifications, and DNA methylation (Ramirez-Prado *et al.,* 2018). Being closely linked, different types of epigenetic changes often act in a self-reinforcing manner to regulate other cellular processes. These mechanisms are crucial for development, cell differentiation, protection against viral genomes and seem to have a critical role in integrating endogenous and environmental signals during the life of a cell or an organism (Vaissière *et al.* 2008; W. Chen *et al.* 2017).

1.4.1. DNA modifications

DNA methylation is a conserved mechanism responsible for regulating gene expression and for having a crucial role in diverse biological processes. Such processes include plant adaptation mechanisms in response to stress conditions, defense against invasion of mobile DNA elements, like transposons, viruses and retroelements, genome management and development process *(Ashapkin et al*. 2016; Moglia *et al.* 2019; Tirnaz & Batley 2019; Gahlaut *et al.* 2020; He *et al.* 2020)

The DNA methylation occurs when a methyl group is added to carbon five (C5) of the DNA strand's cytosine base in both eukaryotes and prokaryotes. Cytosine-5 DNA methyltransferases (C5-MTases) carry out two functions: the recognition of a specific DNA sequence and the catalysis of the transfer of a methyl group from the cofactor/donor S-adenosyl-L-methionine (AdoMet) to carbon 5 in the pyrimidine ring of cytosine residues. Cytosine methylation is essential in gene expression, transposon silencing, and chromosome interaction, as well as in plant growth and development (Vaissière *et al.* 2008; Jeltsch *et al.* 2010; Schübeler 2015; Bej & Basak 2017; Zhang *et al.* 2018). 5-methylcytosine (5-mC) is seen as a marker of epigenetic gene silencing and heterochromatin in plants as well as in mammals. Methylation of DNA only occurs at cytosine residues and can be established by specialized *de novo* DNA methyltransferases enzymes (Pikaard & Scheid, 2014). Due to genome size and complexity and the significant number of transposons, DNA presents the highest methylation rate in plants. Furthermore, it can occur predominantly on any of the following sequence contexts, respectively:

1. CG (cytosine – guanine).
2. CHG (cytosine – H – guanine), which are symmetrical sequences, with H corresponding to A (adenine), T (thymine), or C (cytosine).
3. CHH (cytosine – H – H), an asymmetrical sequence, with H corresponding to A, T, or C.

Dense hypermethylation tends to occur in heterochromatin, which is rich in transposable elements (TEs) and other repetitive DNA sequences, and it plays a preeminent role in silencing gene expression at a transcriptional level (transcriptional gene silencing, TGS). TGS can be triggered by DNA methylation when it is present in gene regulatory regions. Methylation in TEs and repeats have been shown to affect mRNA processing like alternative splicing and alternative polyadenylation (Zhang *et al.,* 2018; Gallego-Bartolomé 2020).

In plants, cytosine methylation is distributed nonrandomly, happening primarily in repetitive regions of the genome that are enriched in transposable elements, centromeric repeats, or arrays of silent 5S or 45S rRNA gene repeats, showing correlations with several modifications on chromatin. Cytosine methylation can also happen at some differentially regulated promoters and within the protein-coding regions of highly expressed genes (Jeltsch & Jurkowska 2010; Pikaard & Scheid 2014; Schübeler 2015). There are two different types of modifications that coexist in plants at two nucleotide bases, cytosine (5-methylcytosine (5-mC)), which is more common in the genome, and adenine (N6-methyladenine (m6A)), which is found in mitochondrial DNA (Bej & Basak 2017; K. J. Wu 2020). However, it is believed that cytosine methylation might influence adenine methylation, even though their interaction is still unknown. Cytosine methylation plays a vital role in the development of plants and survival, turning up transcription regulation in specific development processes as well as in events like vernalization and stress adaptation (Vanyushin & Ashapkin 2011; Bej & Basak 2017; Hoang *et al.* 2018). On the other hand, m6A's possible role in epigenetics in eukaryotes is starting to be unraveled. It has been observed that this epigenetic mark plays crucial roles in transposon's expression, transgenerational inheritance, and stress response (K. J. Wu, 2020).

There are three types of processes that are involved in DNA methylation which are: *de novo* DNA methylation, methylation maintenance, and DNA demethylation.

De novo DNA methylation is catalyzed by domains rearranged methyltransferase 2 (DRM2) through small RNAs (RNA-directed DNA methylation (RdDM) pathway) requiring two plant-specific RNA polymerases, Pol IV and Pol V (Tirnaz & Batley, 2019; Zhang *et al*., 2020). Maintenance of plant DNA methylation depends on the cytosine sequence context and is catalyzed by DNA MTases, which are regulated by different mechanisms. The DNA demethylation is carried out by DNA demethylase enzymes that balance the genome's methylation level and maintain gene expression by removing 5-methylcytosine and replacing it with unmethylated cytosine (Zhang *et al.,* 2018; Tirnaz & Batley 2019; Zhang *et al.,* 2020).

The RdDM pathway, crucial for *de novo* methylation, has been implicated in several regulatory processes in plants. Generally, the methyl groups added by this pathway result in DNA methylation associated with the genetic sequences' transcriptional repression. Also, these changes can often be firmly transmitted to progeny since DNA methylation patterns can be heritable through mitotic and/or meiotic processes. As a result, one notable role of RdDM is the stable, transgenerational suppression of transposable elements (TE) activity. This pathway has also been associated with pathogen defense, abiotic stress responses, and even the regulation of several key developmental transitions (Erdmann & Picard 2020).

Currently, four different types of DNA MTases have been identified in plants and implicated in DNA methylation maintenance, namely: methyltransferase (MET), chromomethylases (CMT), domains rearranged methyltransferase (DRM), and DNA methyltransferase homolog 2 (DNMT2) (Gahlaut *et al.* 2020).

The cytosine methylation in the CG context is the most abundant in plant genomes and is maintained by methyltransferase 1 (MET1), the homolog of animal DNA methyltransferase 1 (DNMT1). Plant-specific chromomethylase 3 (CMT3) is the enzyme responsible for CHG methylation maintenance, together with chromomethylase 2 (CMT2), that also plays an essential role in CHH methylation maintenance in some specific genomic contexts, namely central regions of large transposable elements (Ashapkin *et al.* 2016; Gianoglio *et al*., 2017; Wang *et al.* 2019) (see Figure 2). The DRM is considered to be homologous to animals' DNA methyltransferase 3 (DNMT3) (Gianoglio *et al.*, 2017). Domains rearranged methyltransferase 2 (DRM2) catalyzes CHH methylation at histone H1-containing heterochromatin. Through RdDM pathway, DRM2 is able to maintain CHH methylation at target regions in euchromatin (Bewick & Schmitz, 2017; Gianoglio *et al.*, 2017; Moglia *et al.*, 2019) (see Figure 2). The highly conserved MTase, DNMT2, has a shorter length than other MTases since it does not contain the N-terminal regulatory domains. Despite the fact that this family has a substantial sequence similarity to the MTases and their inclusion of a methyltransferase domain, they do not usually show any DNA MTase activity. Taking this into account, the function of the only DNMT family in plants is still not entirely known (Ashapkin *et al.,* 2016; Gianoglio *et al.,* 2017; Gahlaut *et al.,* 2020). All MTases share a catalytic domain harboring ten small, conserved motifs. However, the four families differ at their N-terminal, reflecting a functional specialization degree (Gianoglio *et al.*, 2017).

On the other hand, DNA demethylation may occur passively or actively (Ruie Liu & Lang, 2020). Passive DNA demethylation is associated with the loss of DNA methylation during DNA replication due to a reduction or inactivation of enzymes responsible for DNA methylation (Li *et al.*, 2018). It also happens when the newly synthesized DNA strand is not appropriately targeted and methylated by DNA MTases (Ruie Liu & Lang, 2020). Active DNA demethylation is mediated by a specific family of plant enzymes, namely Repressor of Silencing 1 (ROS1) and Demeter (DME). Both enzymes are recruited to the target loci and remove 5mC and then cleave the DNA backbone leaving a gap. The resulting single nucleotide gap is then filled with an unmethylated cytosine via the base-excision repair (BER) pathway, wherein apurinic or apyrimidinic site lyase (ARP, APE1L), zinc finger DNA 3′-phosphatase (ZDP), and DNA ligase I (LIG1) are involved (Bochtler *et al.*, 2017; Ruie Liu & Lang, 2020). Until now, this active pathway is less understood in both mammals and plants. Still, it has been gaining increasing attention since it has been shown to be essential for many biological processes in plants, such as in various developmental processes (regulation of seed, stomatal and nodule development, pollen tube formation, and fruit ripening) (Li *et al.*, 2018; Ruie Liu & Lang, 2020). Also, some authors state that defense strategies against pathogen attack are able to generate active DNA demethylation, which is part of the regulatory circuit for gene activation in response to pathogen attack (Weiberg & Jin, 2015; Islam *et al.*, 2018). As an example, some studies declare that some of the RdDM pathway genes are repressed during bacterial infection, leading to demethylation and activation of several defense genes. Also, ROS1, encoding a 5-methylcytosine DNA glycosylase that initiates active DNA demethylation, is repressed after flg22 treatment, and *ros1* mutant exhibits enhanced susceptibility to *Pseudomonas syringae*. This suggests that active DNA demethylation is associated with the regulatory circuit for gene activation in response to pathogen attacks (Yu *et al.,* 2013; Weiberg & Jin, 2015)

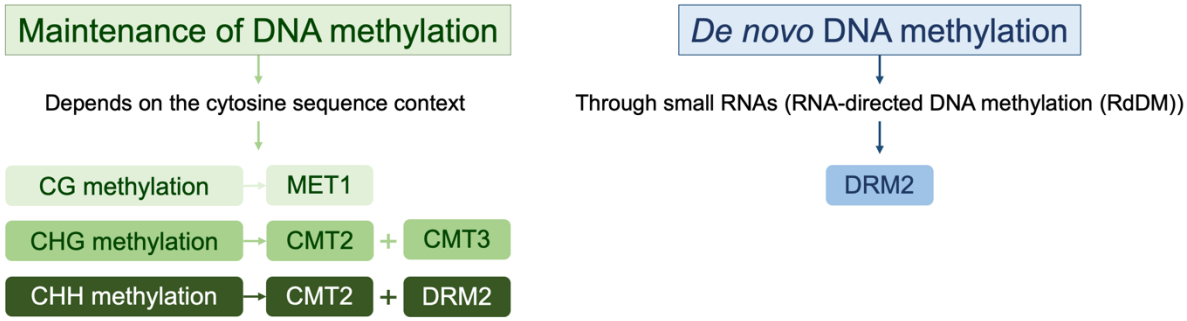


Figure 2 - Scheme of the described functions of enzymes involved in DNA methylation.

1.5. DNA methylation in plant-pathogen interactions: players and mechanisms

Epigenetic modifications are known to have a substantial role in inducing phenotypic diversity, including plant resistance responses through the control of defense-related gene expression levels (Moglia *et al.*, 2019; Tirnaz & Batley, 2019; Gahlaut *et al.*, 2020).

DNA methylation, mediated by C5-MTases, is a widespread epigenetic mark that may cause transcriptome reprogramming during plant-pathogen interactions. However, DNA methylation's specific role in establishing compatible and incompatible interactions (i.e., susceptibility and tolerance, respectively) remains mostly unexplored (Elhamamsy, 2016; Gahlaut *et al.,* 2020; Rambani *et al.,* 2020). Most of the existing studies about the epigenetic control associated with plant-pathogen interactions were conducted in the *Arabidopsis thaliana* - *Pseudomonas syringae* (Pst) pathosystem.

In 2014, studies in *A. thaliana* response against Pst infection allowed to demonstrate that DNA demethylases were associated with expression of stress response genes by targeting promoter transposable element sequences (Le *et al*., 2014). Also, another study showed that exposure to the Pst bacterial pathogen induced a significant level of DNA hypomethylation, which elevated the expression of defense-related genes (Dowen *et al.* 2012; Sahu *et al.* 2013; Elhamamsy 2016).

A recent study presented by Geng *et al*. in 2019 revealed that DNA methylation, especially CHH methylation, is involved in the regulation of defense responses to powdery mildew caused by *Blumeria graminis f. sp. tritici* (Bgt) in wheat diploid progenitor *Aegilops tauschii.* After being infected with Bgt, abundant differentially methylated regions (DMRs) were associated with CHH hypomethylation (Geng *et al.*, 2019). Whole-genome bisulfite sequencing (WGBS) revealed that genes near transposable elements with CHH-hypomethylated DMRs were enriched for “response to stress” functions, involving receptor kinase, peroxidase, and pathogenesis-related genes, suggesting that DNA methylation is involved in the regulation of plant defense responses in crops (Geng *et al.*, 2019).

A year after, another report about the presence of DNA methylation in a compatible and incompatible interaction of tomato with root-knot nematodes was published by Leonetti and his colleagues (Leonetti & Molinari, 2020). In this work, DNA hypomethylation was considered to be the upstream mechanism that triggers the general gene overexpression observed in plant resistance and that DNA hypermethylation and MTase gene activation may give rise to gene silencing induced by nematodes (Leonetti & Molinari, 2020).

Whole-genome bisulfite sequencing (WGBS) of watermelon leaves at different time-points after being infected with cucumber green mottle mosaic virus (CGMMV) was performed by Sun *et al.* (Sun *et al.*, 2019). They noted that the number of differentially methylated regions increased during CGMMV infection, but a clear relationship between DNA methylation and gene expression could not be established since the hypo or hypermethylated genes did not have the correlation with a down-regulation or up-regulation of its expression. Additionally, down-regulation of genes in the RdDM pathway suggested that the reduced RdDM-directed CHH methylation plays a vital role in antiviral defense in watermelon (Sun *et al.*, 2019).

According to these studies, it can be hypothesized that hypomethylation may lead to host defense while hypermethylation may result in higher susceptibility. Also, an epigenetic interplay between the plant host and the pathogen was suggested in some studies since DNA methylation patterns in plants can change when pathogens infect plants. (Hewezi *et al.*, 2018).

A study conducted in 2015, is focused on the interaction between grapevine and a treatment with salicylic acid (SA). It reveals that SA treatments selectively up-regulated expression of some stilbene

synthase (STS), and considerably reduced the cytosine DNA methylation level of these genes in *Vitis amurensis* (Kiselev *et al.,* 2015). As it is known, SA is vital in plant defense, being involved in the activation of defense responses against biotrophic and hemibiotrophic pathogens and in the establishment of systemic acquired resistance (Kiselev *et al.*, 2015).

Although it has already been observed that grapevine presents several features that make it a relevant model for the study of epigenetic mechanisms (Fortes & Gallusci, 2017), no study has been found regarding epigenetic control of defense in grapevine-pathogen interactions.

1.6. Main Aims

Considering that DNA methylation may be one of the epigenetic modifications associated with plant defense against pathogens, this work aims to characterize the *Vitis vinifera* DNA methyltransferase gene family and start to unravel its role in the establishment of compatible and incompatible interactions between grapevine and *Plasmopara viticola*.

To achieve this aim, a genome-wide characterization of DNA methyltransferases gene family was conducted, candidate genes were selected, and expression analysis was carried out in *V. vinifera* cv 'Trincadeira' (susceptible) and *V. vinifera* cv 'Regent' (tolerant) at 6, 24, 48, 72 hours post inoculation and 5 days post inoculation with *P. viticola*. DNA global methylation patterns were also determined and correlated with DNA methyltransferase gene expression.

2. Materials and Methods

2.1. Bioinformatic characterization

2.1.1. Grapevine methyltransferases identification and characterization

Methyltransferases (MTase) genes and putative protein sequences have been already identified in model plants, namely in *Arabidopsis thaliana* (thale cress) (Pavlopoulou & Kossida 2007; Ashapkin *et al.* 2016), *Oryza sativa* (rice) (Pavlopoulou & Kossida 2007; Ahmad *et al.* 2014), *Nicotiana tabacum* (tobacco) (Pavlopoulou & Kossida 2007), *Solanum lycopersicum* (tomato) (Teyssier *et al.* 2008; Cao *et al.* 2014) and *Zea mays* (maize) (Pavlopoulou & Kossida 2007; Qian *et al.* 2014).

DNA methyltransferases (MTases) protein sequences of the model organisms mentioned above were used as queries in UniProt (https://www.uniprot.org/) and NCBI databases (https://blast.ncbi.nlm.nih.gov/Blast.cgi). MTases domain sequences, i.e., DNMT1-RFD (pfam12047), BAH (pfam01426), Chromo (pfam00385), DCM (COG0270), and AdoMet_MTases (pfam07757), were also used as query.

For the identified grapevine MTases protein sequences, the presence and distribution of conserved domains were confirmed by Pfam (https://pfam.xfam.org/) analysis. The mRNA sequences were retrieved for the selected grapevine MTases.

2.1.2. Bioinformatic analysis of *V. vinifera* DNA methyltransferases: sequence properties, subcellular location, and chromosomal localization

Molecular weight (Mw) and theoretical isoelectric point (pI) were predicted with the pI/Mw tool from ExPASy (https://web.expasy.org/compute_pi/).

TargetP 2.0 (http://www.cbs.dtu.dk/services/TargetP/), Localizer (http://localizer.csiro.au/) (Sperschneider *et al.*, 2017), SignalP-5.0 (http://www.cbs.dtu.dk/services/SignalP/) (Almagro Armenteros *et al.*, 2019), Nuc-PLoc (http://www.csbio.sjtu.edu.cn/bioinf/Nuc-PLoc/) (Shen & Chou 2007), NucPred batch predictor (https://nucpred.bioinfo.se/cgi-bin/batch.cgi) (MacCallum *et al.* 2007), NetNES 1.1 Server (http://www.cbs.dtu.dk/services/NetNES/) (Cour *et al.* 2004), Predotar (https://urgi.versailles.inra.fr/Tools/Predotar) and Wolf Psort (https://wolfpsort.hgc.jp/), were used for subcellular location prediction.

Biological function prediction for grapevine MTases was determined using the Blast2GO software (https://www.blast2go.com/). Open Reading Frame (ORF) length was predicted using the ORFfinder tool from NCBI (https://www.ncbi.nlm.nih.gov/orffinder/). The chromosomal location and position of the MTases sequences, as well as the number of exons, introns, and gene length, were predicted using Genome Data Viewer tool from NCBI (https://www.ncbi.nlm.nih.gov/genome/gdv/). MTase sequences with a chromosomal location near to “Resistance to *Plasmopara viticola*” (RPV) loci on grapevine genome were selected. All data were manually curated and compiled.

2.1.3. Alignment of protein sequences and phylogenetic analysis

The protein sequences obtained from the NCBI database were aligned using the CLUSTAL X, with version 2.0 (http://www.clustal.org/clustal2/) (Larkin *et al.*, 2007). Alignment quality was verified through MAFFT software with the L-INS-I option version 7 (http://mafft.cbrc.jp/alignment/software/) (Katoh & Standley 2013).

In order to compare the methyl family groups, a preliminary *neighbor-joining* phylogenetic tree was constructed using MEGA-X software (v10.2.2; https://www.megasoftware.net), with the following standard parameters: protein substitution model *Maximum Composite Likelihood*; bootstrap 1000 interactions (Kumar *et al.*, 2018). The identified methyltransferase sequences of *V. vinifera* were used, as well as the known DNA methyltransferases sequences from *A. thaliana*, *O. sativa,* and *Z. mays*. A phylogenetic tree was created using the FIGTree software (v1.4.3, http://tree.bio.ed.ac.uk/software/figtree/). Lastly, another phylogenetic tree was generated with only DNA methyltransferases sequences from *V. vinifera*, using the same parameters. This analysis may evidence some relationships between *V. vinifera* DNA methyltransferases.

2.2. Expression analysis

2.2.1. Plant material

Two *V. vinifera* genotypes, 'Regent' and 'Trincadeira', were used as models of an incompatible and a compatible interaction with *Plasmopara viticola*, respectively.

The cultivar 'Regent' was bread by multiple introgressions from tolerant wild genotypes. It presents an RPV3 loci, that revealed several candidates genes, which might be responsible for the expression of resistance against *P. viticola*. Regent also displays a high degree of tolerance to both downy and powdery mildew (Figueiredo *et al.,* 2008; VIVC, 2020). 'Trincadeira' is a Portuguese elite grapevine cultivar widely used for the production of high quality wine, and it is highly susceptible to these pathogens (Welter *et al.* 2007; Figueiredo *et al.* 2008; VIVC, 2020)

Plant material, mock and inoculated with the pathogen, as described in Figueiredo *et al.* (2012), was already available in the lab. Briefly, the third to the fifth fully expanded leaves beneath the shoot apex were harvested at 6, 24, 48, 72 hours post inoculation (hpi) and 5 days post inoculation (dpi), immediately frozen in liquid nitrogen and stored at −80 °C. For each genotype and condition (inoculated and mock-inoculated), three independent biological replicates were collected, each consisting of a pool of ten leaves from three different plants.

2.2.2. RNA extraction and cDNA synthesis

Total RNA was extracted from *V. vinifera* cv. 'Regent' and 'Trincadeira' leaves, mock and inoculated, using the Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, USA), according to manufacturer's

instructions. Briefly, 500 µL of the Lysis Solution supplemented with 2-mercaptoethanol (10 µL) was added to 1 g of tissue powder, vortexing for 30 seconds. Samples were incubated for 5 minutes at 56ºC and centrifuged at maximum speed for another 5 minutes to pellet cellular debris. Clear supernatant was transferred into a filtration column, followed by maximum speed centrifugation for 7 minutes. 250 µL of Binding Solution were added to the clarified lysate, mixed immediately and thoroughly by pipetting at least 5 times. After another centrifugation step, 300 µL of Wash Solution 1 were added into the column, followed by centrifugation. Genomic DNA (gDNA) was digested with the On-Column DNase I Digestion Set (Sigma-Aldrich, USA), by adding 40 µL of DNase, followed by an incubation of 30-40 minutes. 500 µL of Wash Solution 1 were added and centrifugated. Then, the flow-through liquid was discarded, and 500 µL of Wash Solution 2 were added. This step was performed twice. Then, 30 µL of Elution Solution were added twice directly onto the center of the binding matrix inside the column, and RNA isolated.

Using a spectrophotometer (NanoDrop-1000, Thermo Scientific), purity and concentration of extracted RNA were assessed through OD measurements at $A_{260/280\ nm}$ and $A_{260/230\ nm}$, while its integrity was monitored by agarose gel electrophoresis.

All samples were tested for gDNA contamination by quantitative real-time polymerase chain reaction (qPCR) using 1 µL of crude RNA by targeting the reference gene *elongation factor 1-alpha* (*EF1α*) (Vandesompele *et al.*, 2002).

Complementary DNA (cDNA) was synthesized from 2.5 µL of total RNA, using RevertAid®H Minus Reverse Transcriptase (Fermentas, Ontario, Canada), anchored with 1 µL oligo$(dT)_{23}$ primer (100 µM) (Fermentas, Ontario, Canada) by incubating at 70ºC for 10 minutes. Then, 7 µL of a prepared mix containing 4 µL of Reaction Buffer (5X), 2 µL of dNTP Mix (10 mM), 0.5 µL of RevertAid H Minus M-MuLV Reverse Transcriptase (200U/µL) as well as0.5 µL RiboLock RNase Inhibitor (20U/µL), were added to the solution and cDNA synthesis occurred during 90 minutes at 42ºC. A final denaturation step was added by incubating the mixture for 10 minutes at 70 ºC as described previously (Figueiredo *et al*., 2017).

2.2.3. Quantitative Real-Time PCR (qPCR)

Using the iTaq™ Universal SYBR® Green Supermix (Bio-Rad, USA), qPCR experiments were conducted according to the manufacturer's instructions. Briefly, 5 µL of SYBR Green 5x and 0.5 µL of each primer (10 µM) were used in 10 µL volume reactions, together with 1 µL of cDNA as template. A control without a cDNA template (NTC) was included in each set of reactions. Three biological replicates and two technical replicates were used for each sample. Primer sequences and amplification details are provided in Table 1. Reactions were carried out in a StepOne™ Real-Time PCR system (Applied Biosystems, USA). To confirm single product amplification and the existence of non-specific PCR products, a dissociation curve analysis was performed. Gene expression (fold change) for both compatible ('Trincadeira' versus mock-inoculated control samples) and incompatible interactions ('Regent' versus mock-inoculated control samples) was calculated as described in (Hellemans *et al.*,

2008). *Elongation factor 1-alpha* (*EF1α*) and *SAND family protein* (*SAND)* genes were used for expression data normalization (Monteiro *et al.*, 2013).

Table 1 - Reference and interest genes primer sequences, amplicon length, amplification efficiency, annealing, and melting temperature are represented.

Adopted identifier NCBI Accession Number		**Primer sequence**	**Amplicon length (bp)**	**Amplification efficiency (E)**	**Ta (°C)**	**Tm (°C)**
Reference genes		**(5'- 3')**				
EF1α (*Elongation factor 1-alpha*) XM_002284888.2		Fw: GAACTGGGTGCTTGATAGGC Rev: ACCAAAATATCCGGAGTAAAAGA	149	2.01	60	79.95
SAND (*SAND family protein*) XM_002285134.2		Fw: CAACATCCTTTACCCATTGACAGA Rev: GCATTTGATCCACTTGCAGATAAG	76	2.04	60	78.86
DNA MTase	Family Group					
VviDNMT1 XM_002282418.4	DNMT	Fw: CAACTTTCGTGAAGGCACTG Rev: GCTTCTCGAATGATGGGTG	69	1.98	58	79.65
VviDRM2 δ XM_010651350.1	DRM	Fw: CAGAACCCATGCACTTACTC Rev: AACACGGACAAACTCACAAAA	102	1.98	58	76.48
VviDRM1 XM_010662592.2	DRM	Fw: GCAAGATGGGGAAGGAAACA Rev: GACAGTTCTCAAGAGCCGAG	71	2.07	60	75.23
VviCMT2 ε XM_010666729.2	CMT	Fw: GTTAACTTACCATCACCATACC Rev: TACCATCTTCTAATCCTCCTGT	119	-	62	-
VviCMT2 α XM_010663837.2	CMT	Fw: GGAATTGCCATCTGGAAAGC Rev: GCAGTGAGGGTCAGGTTTAG	136	1.95	60	79.20
VviCMT1 β XM_010653042.2	CMT	Fw: CCCCCAAAATTCCCTAACAT Rev: TCCACCAAAAGAGACATCCC	103	1.94	58	75.87
VviCMT1 α XM_002275896.3	CMT	Fw: GGCTTTCCTGACTGCTACAA Rev: CTAACGGGTGGTCATCACAG	145	1.99	60	81.15
VviMET1 XM_002267164.3	MET	Fw: GCACAGGCAGATTGGAAATG Rev: AAGATGGACCTCTTGCTGTC	93	2.03	60	78.98
VviMET2 α XM_002267248.4	MET	Fw: TCCCAAAGGCACCCAAAATAG Rev: CACCTCACGGATGTCAGAAG	116	1.97	62	77.85

2.2.4. Genomic DNA (gDNA) extraction

The innuPREP Plant DNA Kit (Analytik Jena AG, Germany) was used to isolate genomic DNA from frozen samples *of V. vinifera* cv. 'Regent' and 'Trincadeira' leaves, mock and inoculated, according to the manufacturer's instructions. Summarily, 0.1 g of leaf powder was used and 400 µL of lysis solution OPT were added. The mixture was vortexed for 5 seconds. After being incubated for 30 minutes at 65ºC, 100 µL of precipitation buffer were added, mixed in the vortex for 5 seconds, followed by incubation at room temperature for 5 minutes. After centrifugation at maximum speed for 5 minutes, clear supernatant was transferred onto a prefilter in a receiver tube and centrifuged. 4 µL of RNase A solution were added to the previously filtered solution and incubated for 5 minutes at room temperature. A total of 200 µL of binding solution SBS were added to the obtained lysed sample, creating a homogeneous solution, and allowing the binding of DNA to a spin filter column. New centrifugation steps were performed, ending with two washes with 650 µL of washing solution MS. 50 µL of elution buffer were added and samples were incubated at room temperature for 1 minute.
Genomic DNA quality was evaluated at $A_{260/280\ nm,}$ and $A_{260/230\ nm}$ using a spectrophotometer (NanoDrop-1000, Thermo Scientific, USA) and its integrity was assessed on an agarose gel electrophoresis.

2.2.5. Detection of global 5-methylcytosine (5-mC) in DNA

The concentration of 5-methylcytosine (5-mC) present in gDNA was quantified using the 5-mC DNA ELISA Kit (Zymo Research, California, USA), measured according to the manufacturer's instructions. Briefly, 100 µL of DNA samples were denatured at 98ºC for 5 minutes in a thermal cycler, being immediately transferred into ice for 10 minutes. Denatured DNA was then coated onto a plate by incubating at 37ºC for 1 hour. The plate was washed with 5-mC ELISA buffer three times. 5-mC ELISA buffer was added to each well, and the plate was after covered with aluminum foil and incubated at 37ºC for another 30 minutes. The kit features a unique anti-5-methylcytosine monoclonal antibody, sensitive and specific for 5-mC, that was added to the plate mixed with a secondary antibody (with the horseradish peroxidase reporter – HRP). 100 µL of the antibody mix were added to each well, the plate was covered with foil and incubated again at 37ºC for 1 hour.
The antibody mix was discarded from the wells, and each well was washed three times with 5-mC ELISA buffer. Subsequently, an HRP developer was added to each well and color development occurred. All samples were assayed in duplicate. The absorbance was read at 405 nm after approximately 20 minutes of color development, and a standard curve was plotted. % 5-mC in the DNA samples (mock and inoculated) was quantified from the standard curve generated with specially designed controls included with the kit.

2.2.6. Statistical analysis

Statistical significance (*p-value* < 0.05) of gene expression between target and control samples from the two genotypes was determined by the Mann-Whitney U test using IBM® SPSS® Statistics version 23.0 software (SPSS Inc., USA).
In addition, statistical analysis was also performed on DNA methylation data, using a Welch's t test from GraphPad Prism software version 8.2.1.

3. Results and Discussion

3.1. Characterization of DNA methyltransferases in *Vitis vinifera*

3.1.1. Grapevine methyltransferases identification

Eukaryotic DNA methyltransferase protein sequences from *A. thaliana*, *O. sativa*, *N. tabacum*, *S. lycopersicum,* and *Z. mays*, as well as methyltransferase domains, i.e., DNMT1-RFD (pfam12047), BAH (pfam01426), Chromo (pfam00385), DCM (COG0270) and AdoMet_MTases (pfam07757), were used as a query in NCBI to retrieve both *Vitis vinifera* DNA methyltransferases mRNA and protein sequence identifiers.

Nine grapevine methyltransferases genes were identified, and 17 methyltransferases proteins were predicted due to alternative splicing (see Supplementary Table 1). Alternative splicing of pre-mRNA is a conserved mechanism that is known to increase transcriptomic plasticity, proteomic complexity, and phenotypic diversity through the production of multiple mRNA isoforms from just a single gene (Vitulo *et al.* 2014; Laloum *et al.,* 2018). These events have been observed in *A. thaliana*, *Z. mays*, *O. sativa,* and *V. vinifera* and have been linked to plant development and stress responses (Vitulo *et al.,* 2014; Thatcher *et al.,* 2016). To date, alternative splicing events were identified in the MET subfamily from *O. sativa* (*OsMET1*) (Yamauchi *et al.*, 2008) and in *DRM* genes from strawberry (Chang *et al.*, 2009). The number of C5-MTases genes found in *V.vinifera* is comparable to that of other plants, such as *A. thaliana* (11)*, O. sativa* (10)*,* and *Z. mays* (8) (Pavlopoulou & Kossida 2007; Qian *et al.* 2014; Ahmad *et al.* 2014; Li *et al.* 2018).

V. vinifera C5-MTase genes were distributed among 8 of the 19 grapevine chromosomes (see Figure 3). In *Z. mays*, the 8 C5-MTase genes were found to be distributed equally across 6 of the 10 chromosomes (Qian *et al.*, 2014). Regarding *S. lycopersicum* C5-MTases genes, Cao and co-workers showed that the 7 genes were dispersedly located in 7 of the 12 tomato chromosomes (Cao *et al.,* 2014). By analyzing the *Vitis vinifera* results with those obtained in these two organisms, we can conclude that the distribution of C5-MTases genes is much wider and more dispersed in maize and tomato than in grapevine.

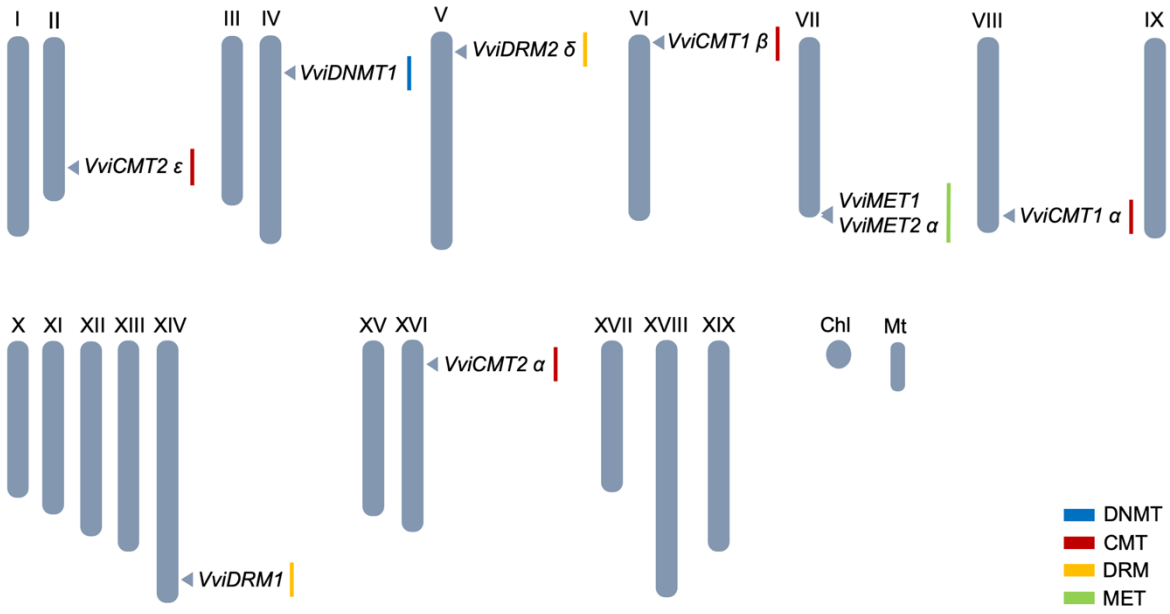


Figure 3 - Prediction of the MTases genes' location in the grapevine chromosomes using Genome Data Viewer tool from NCBI. Proposed Vitis MTases nomenclature is shown in each chromosome. DNA methyltransferase homolog (DNMT), chromomethylases (CMT), domains rearranged methyltransferase (DRM) and methyltransferase (MET) are represented with different colors. Chloroplast (Chl) and mitochondrial (Mt) DNA are also symbolized.

3.1.2. Phylogenetic analysis of grape methyltransferases

To understand the phylogenetic relationships between grapevine DNA methyltransferases, an unrooted neighbor-joining phylogenetic tree was constructed using full-length protein sequences of the 17 C5-MTases from *V. vinifera* and other plant species, i.e., *A. thaliana*, *O. sativa,* and *Z. mays* with 11, 5, and 5 proteins, respectively (see Figure 4).

Four subfamilies were observed in the tree with well-supported bootstraps values, namely methyltransferase (MET), chromomethylase (CMT), domains rearranged methyltransferase (DRM), and DNA methyltransferase homolog 2 (DNMT2), which is consistent with previous studies (Pavlopoulou & Kossida 2007; Cao *et al*. 2014; Qian *et al.* 2014; Du 2016; Gahlaut *et al.* 2020).

Analyzing the obtained results, VviDNMT1 was grouped into the first subfamily with only one protein, sharing a remarkable similarity to the DNMT2-like class in Arabidopsis, whose function is still unknown (Pavlopoulou & Kossida 2007; Ashapkin *et al.* 2016; Wendte & Schmitz 2018).

The class of chromomethylases, found only in plants (Bewick *et al*., 2017), can be grouped into two clades, VviCMT1 (CMT1/CMT3/ZmMET2 in Arabidopsis and maize, respectively) and VviCMT2 in grapevine. In *A. thaliana*, this division is associated with sequence specificity, since CMT2 methylates cytosines mainly in the CHH context and CMT3 methylates cytosines mostly in the CHG context. Although AtCMT1 has no function, according to Wendte & Schmitz, its retention across angiosperms suggests that one is yet to be discovered (Wendte & Schmitz, 2018). Some studies carried out in *Z.*

mays have shown that the specificity for CHG versus CHH contexts, is not as robust as observed in *A. thaliana* since ZmMET2a and ZmMET2b are responsible for methylating both CHH and CHG contexts (Li *et al.* 2014; Qian *et al.* 2014; Wendte & Schmitz 2018).

Considering the MET subfamily, VviMET1 and VviMET2 were identified, sharing significant similarity with *A. thaliana* (AtMET2a, AtMET2b, and AtMET3) and *O. sativa* proteins (OsMET1-1 and OsMET1-2). Orthologous to mammalian DNMT1, the MET proteins maintain the methylation status by recognizing hemi-methylated CG sites and methylating the newly synthesized DNA strand during DNA replication (Wendte & Schmitz 2018; Moglia *et al.* 2019).

Belonging to the DRM subfamily, VviDRM1 shared higher similarity with ZmMET3a and ZmMET3b, while VviDRM2 exhibited a higher similarity to AtDRM3, OsDRM1, and ZmMET3c. This subfamily is responsible for catalyzing *de novo* methylation in all sequence contexts, although CHH methylation tends to be first (Wendte & Schmitz 2018) (Moglia *et al.,* 2019). DRM2 is also known to maintain DNA in CHH methylation (Leonetti & Molinari 2020).

A phylogenetic tree with only *Vitis vinifera* protein sequences was created to classify the Vitis sequences within each group (see Figure 5). Four groups were defined as previously, and the CMT group contains seven proteins (VviCMT1 α, VviCMT1 β, VviCMT2 α, VviCMT2 β, VviCMT2 γ, VviCMT2 δ, and VviCMT2 ε). DNMT group contains a single protein in grapevine, which is VviDNMT1. The evolutionary branch of DRM shows up to six protein sequences (VviDRM1, VviDRM2 α, VviDRM2 β, VviDRM2 γ, VviDRM2 δ, and VviDRM2 ε) and MET group comprises three proteins (VviMET1, VviMET2 α and VviMET2 β).

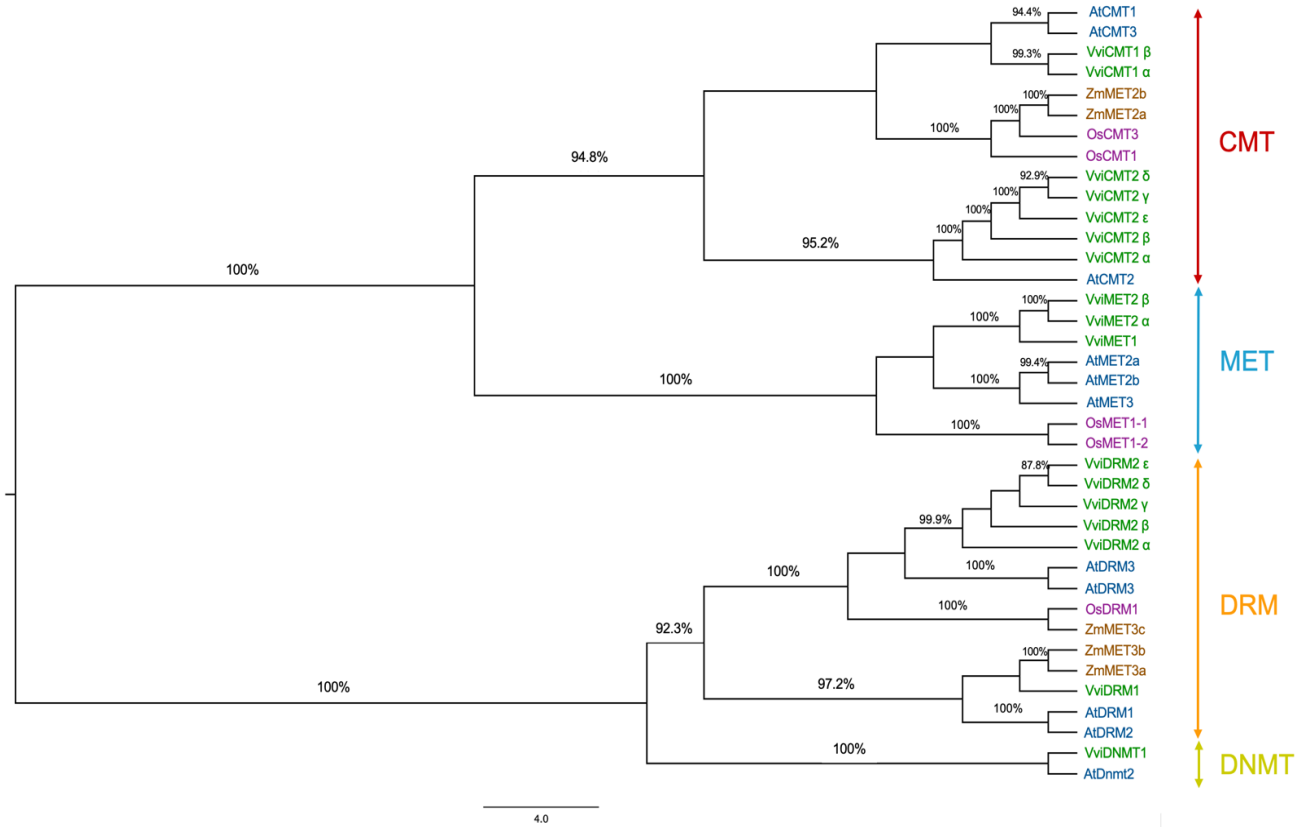


Figure 4 - Phylogenetic tree with *Vitis vinifera* protein sequences compared with proteins from other plant species, i.e., *A. thaliana, O. sativa* and *Z. mays*. The four characteristic MTases groups are identified: methyltransferases (MET), chromomethylase (CMT), domains-rearranged methyltransferases (DRM) and DNA methyltransferase homologue (DNMT). The numbers above branches show bootstrap values (values below 70% are not shown, since are not relevant). Scale bar represents the number of changes per site (estimated branch length).

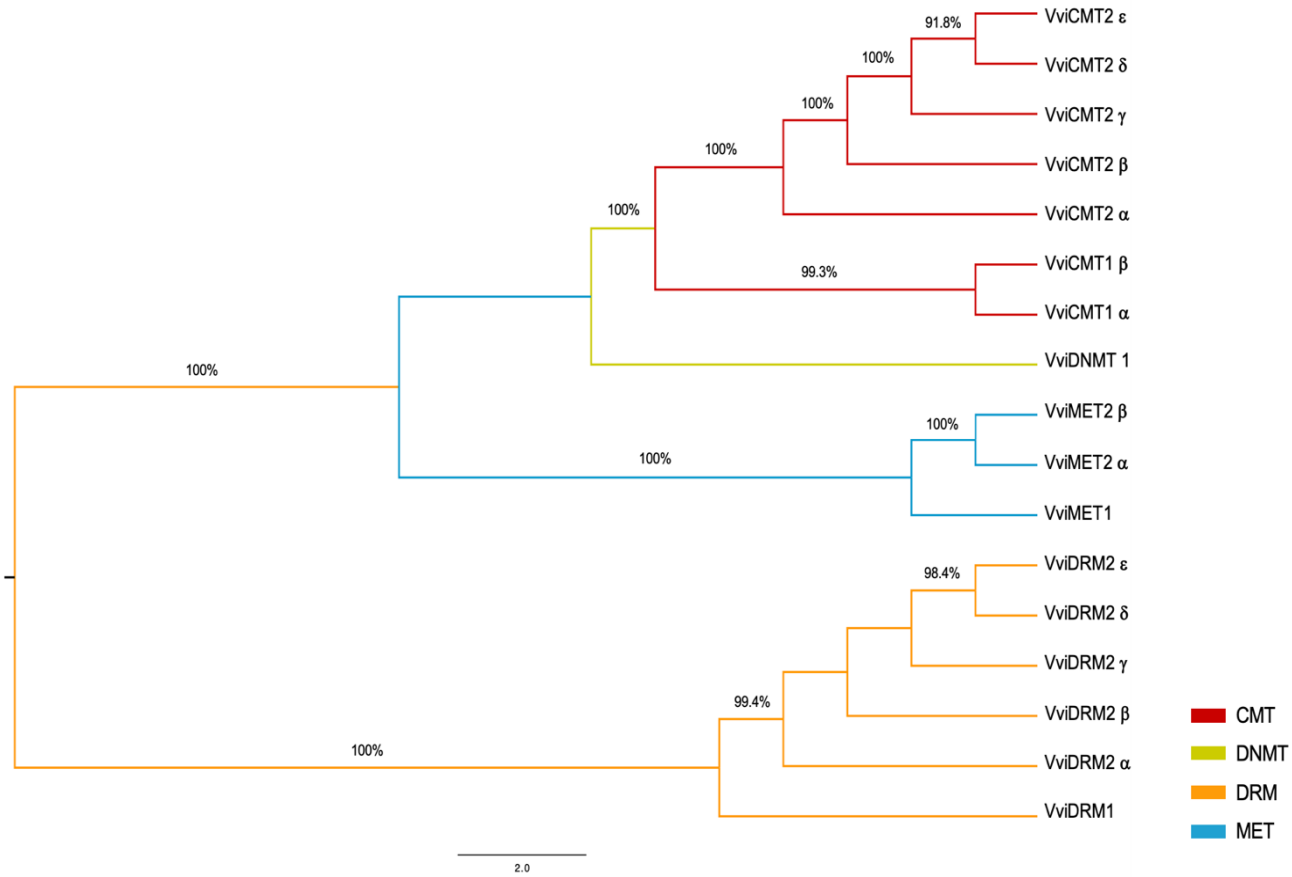


Figure 5 - Phylogenetic tree with *Vitis vinifera* protein sequences. The four characteristic MTases groups are identified: methyltransferases (MET), chromomethylase (CMT), domains-rearranged methyltransferases (DRM) and DNA methyltransferase homologue (DNMT). The numbers above branches show bootstrap values (values below 70% are not shown, since are not relevant). Scale bar represents the number of changes per site (estimated branch length).

3.1.3. Grapevine methyltransferase proteins: protein structure and properties

For the identified grapevine DNA methyltransferases protein sequences, molecular weight (Mw) and the isoelectric point (pI), the identification of the conserved domains, the subcellular location, and function were determined (see Supplementary Table 1).

Our results showed that *Vitis vinifera* MTases proteins have a broad spectrum of molecular weights, between 44.85 and 176.94 kDa, which is in agreement with the already described for other plant MTases (Sharma *et al.* 2009; Qian *et al.* 2014; Li *et al.,* 2018). Of the 17 MTases analyzed, we can verify that about 47.05% of the proteins have a molecular weight between 44 and 100 kDa. This group includes all proteins from VviDRM and VviDNMT1 subfamilies and VviCMT1 α. The remaining 52.94% of proteins corresponding to the VviMET and VviCMT subfamilies, have values between 100 and 177kDa. Very similar results have been observed in *A. thaliana*. 54.55% of protein's molecular weight is within the range of values 43.7 and 94.9 kDa. The remaining percentage includes 145.0 and 174.8 kDa values with all MET proteins and one protein from the CMT group (Li *et al.,* 2018).

Analyzing the isoelectric point values, grapevine MTases present a theoretical pI between 4.93 and 8.84, predicted with the pI/Mw tool from ExPASy. The same range of values was reported for *A. thaliana,* which varies between 4.87 and 8.64 (Li *et al.* 2018). Considering the protein sequences from rice, the pI values are not entirely in the same spectrum, having 5.14 as the lowest value and 6.65 for the highest pI. The same occurs in maize, which has a range of values between 5.13 and 6.58 (Qian *et al.*, 2014). These differences may be associated to different functions in DNA cytosine methylation.

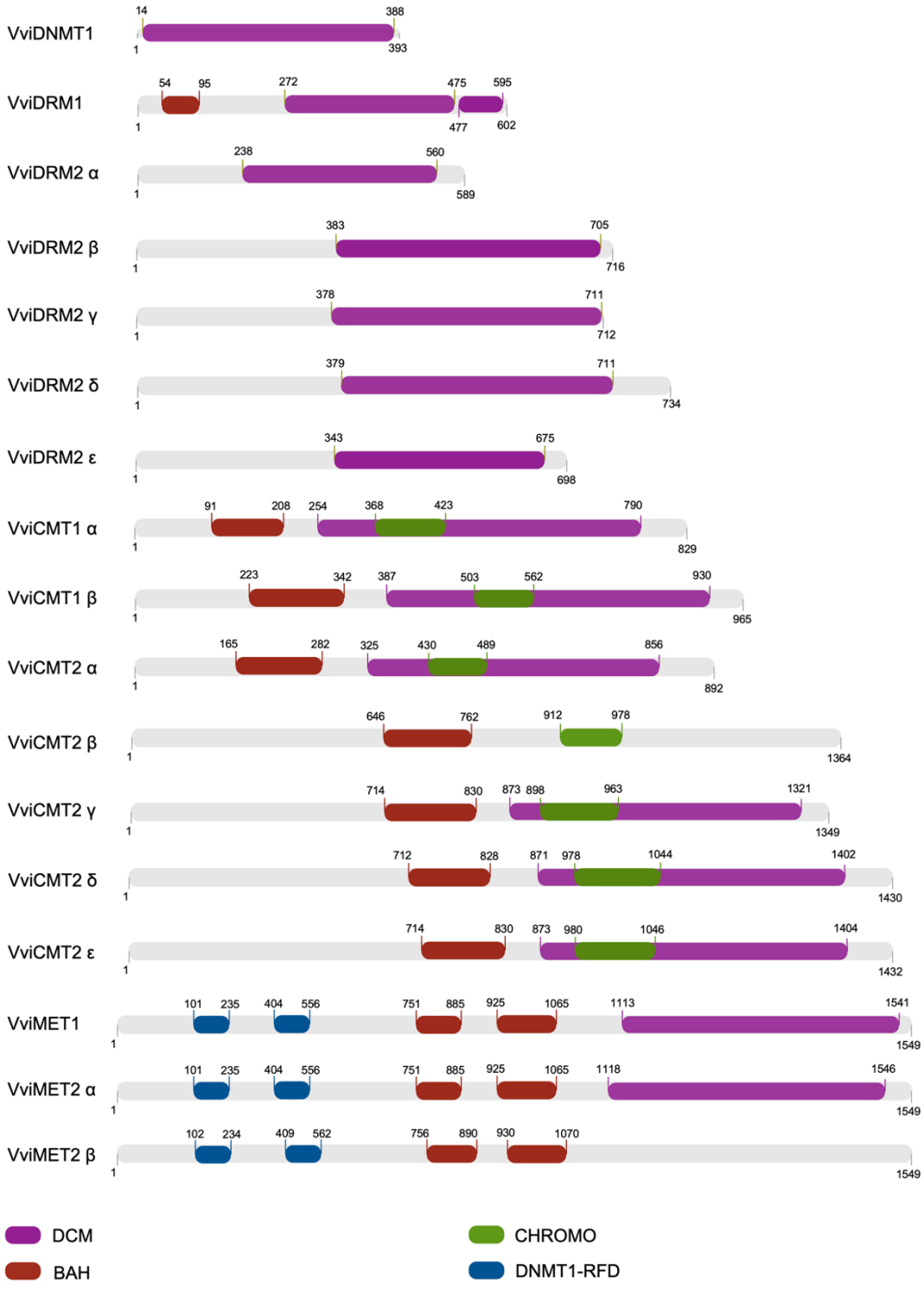


Figure 6 - Domain prediction results of *Vitis vinifera* MTases, obtained through NCBI and InterPro database.

The presence and distribution of the conserved domains in *V. vinifera* sequences were analyzed by Pfam and NCBI pBLAST tools. The N-terminals have diverse combinations of conserved domains among subfamilies. DNMT1-RFD (PF12047), BAH (PF01426), Chromo (PF00385) and DCM (COG0270) were observed in *V. vinifera* MTases proteins.

Generally, the CMT and MET families that presented greater domain diversity - between 2 and 3 conserved domains. DRM and DNMT families present only one conserved domain, except for VviDRM1, that possesses 3 conserved domains. Our results are in agreeance with those obtained by Li *et al*. regarding the MET and CMT families, as all MTases in VviMET enzymes possess two BAH domains while the MTases in VviCMT have only one. In addition, we note that VviMET enzymes are characterized by having two domains of each, BAH and DNMT1-RFD, while VviCMT possess one BAH domain and one CHROMO domain. Present in almost every protein identified, the BAH domain was detected in 11 proteins (VviDRM1, VviCMT1 α, VviCMT1 β, VviCMT2 α, VviCMT2 β, VviCMT2 γ, VviCMT2 δ, VviCMT2 ε, VviMET1, VviMET2 α and VviMET2 β), as well as the DCM domain that was predicted in almost all of the analyzed proteins, except for one (VviMET2 β). Analyzing the domain prediction results, not all grapevine MTases have all 4 domains simultaneously, as is shown in Figure 6 and Supplementary Table 2.

Prediction of the subcellular location of grapevine MTases was performed using different software packages. Fourteen MTases proteins from *V. vinifera* were predicted to be located in the nucleus (VviDNMT1, VviDRM1, VviDRM2 δ, VviDRM2 β, VviDRM2 γ, VviDRM2 ε, VviDRM2 α, VviCMT1 α, VviCMT2 α, VviCMT2 ε, VviCMT2 δ, VviCMT2 γ, VviCMT2 β and VviCMT1 β), one in the chloroplast (VviMET1) and the remaining proteins were predicted to be located in the vacuole (VviMET2 α and VviMET2 β), as shown in Figure 7. In accordance with this, some studies revealed that the majority of C5-MTases in rice, tomato, wheat, and peanut were reported to be in the nucleus and/or cytoplasm (Malik *et al.*, 2012; Cao *et al.*, 2014; Wang *et al.*, 2016; Gahlaut *et al.*, 2020). Gahlaut and coworkers in 2020 revealed that the nucleus localization of many DNA methyltransferases might indicate their role in DNA methylation, replication, and transcriptional regulation. He also stated that it is possible that C5-MTases, which are predicted to be in the cytoplasm and nucleus, might function in the stress-management machinery in the cell (Gahlaut *et al.*, 2020).

Interestingly, in *Ricinus communis*, two members of the DRM family (DRM1 and DRM3) were predicted to be in the nucleus, chloroplast, and mitochondria, suggesting that this MTase family might be dual-targeted to mitochondria and chloroplasts to regulate gene expression through DNA methylation modification in organelle genomes (Victoria *et al.*, 2018). A dual localization may allow some of these proteins to act as direct regulators for the coordinated expression of the mitochondrial, chloroplast, and nuclear genomes in response to environmental and developmental signals (Victoria *et al*., 2018). Another work with the same plant material suggested that the presence of these MTases in the chloroplasts might be related to DNA methylation since their samples were from young leaf tissues (He *et al.*, 2017). Also, the chloroplast has been recognized for playing a significant role in the plant's stress responses, since it has the presence of DNA and MTases methylate in this complex molecule (Ding *et al.*, 2019). Once immune responses are activated, chloroplasts are the source of multiple defensive

signals, including reactive oxygen species (ROS) (Ding *et al.*, 2019). Recent findings have observed that chloroplasts cluster around the nucleus during plant-pathogen interactions, suggesting a pivotal role of this positioning in plant defense, and identifies ROS as being sufficient and necessary for the initiation of this response (Ding *et al.*, 2019).

Blast2GO was used to determine possible roles of MTases, GO terms associated to DNA methylation, methyltransferase activity or protein and chromatin binding activities were found which is in accordance to the MTases general role (Finnegan & Kovac, 2000; Pavlopoulou & Kossida, 2007; Li *et al*., 2014).

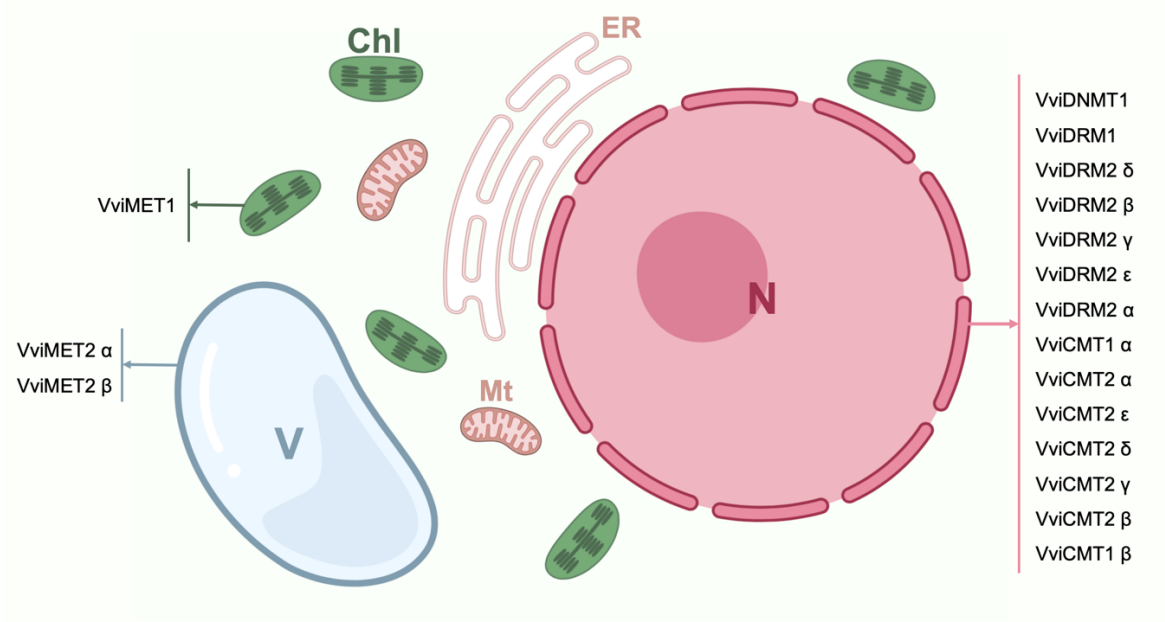


Figure 7 - Predicted subcellular localization of *Vitis vinifera* MTases. V - Vacuole; N - Nucleus; Chl - Chloroplast; Mt - Mitochondria; ER - Endoplasmic Reticle. Created with BioRender.

3.1.4. Gene structure analysis: ORF length and exons/introns

A gene structure analysis was made through the determination of the ORF length as well as the number of exons and introns. The length of ORFs of MTases grapevine genes varies from 1182 bp to 4710 bp (see Supplementary Table 3). This scale of values is very similar to that observed in *A. thaliana*, which show a minimum ORF length of 1152 bp corresponding to 383 aa and a maximum value of 4638 bp corresponding to 1545 aa (Li *et al.* 2018) and *Z. mays* with 1074 bp and 4611 bp of ORF length (Qian *et al*., 2014).

The organization of exons/introns of *V. vinifera* MTases genes was analyzed. As shown in Supplementary Table 3, the number of exons in the *V. vinifera* MTases varied from 10 to 25. We observed that genes belonging to VviMET and VviDRM subfamily had around 11 to 12 exons, the VviCMT subfamily displayed 21 to 25 exons, and the VviDNMT1 subfamily carried 10 exons. A similar pattern was observed in *A. thaliana* MTases genes, which varied from 7 to 22 exons (Li *et al.,* 2018).

3.1.5. Methyltransferases potentially involved in grapevine immunity

Considering that MTases may play a role in grapevine defense against *P. viticola*, the association of their chromosomal location with "Resistance to *Plasmopara viticola*" (RPV) loci was assessed. To date, 27 quantitative trait loci (QTL) associated with *P. viticola* resistance have been identified (Possamai *et al.*, 2020). Our results showed that only two MTases genes were located near to RPV loci. At 4.5 Megabase pairs (Mbp) in chromosome 4, the LOC109121395 (adopted identifier: *VviDNMT1*) gene was located near Rpv4, found at 4.7 Mbp. The LOC100258788 (adopted identifier: *VviCMT1 β*) gene located at 1.2 Mbp in chromosome 6 was near the Rpv20, found at 0.9 Mbp (see Table 2). Following Divilov *et al.* (2018) research, QTL on chromosome 6 may be associated with sporulation and hypersensitive response (Divilov *et al.*, 2018).

Table 2 - Chromosomal location of grapevine MTases genes and chromosomal location of 'Resistance to *Plasmopara viticola* – RPV' sites

Adopted identifier	Nucleotide	Gene ID	Chromosome		RPV	
			Chromosome	Position (Mbp)	RPV	Position in the chromosome (Mb)
VviDNMT1	XM_002282418.4	LOC109121395	4	4.5	Rpv4	4.7
VviDRM1	XM_010662592.2	LOC100254189	14	27.5	Rpv19	29.5
VviDRM2 δ	XM_010651350.1	LOC100247823	5	2.4	Rpv11	4.1
VviCMT1 α	XM_002275896.3	LOC100252100	8	20.5	Rpv17	11.7
VviCMT1 β	XM_010653042.2	LOC100258788	6	1.2	Rpv20	0.9
VviCMT2 α	XM_010663837.2	LOC100249359	16	2.7	-	-
VviCMT2 ε	XM_010666729.2	LOC100255190	2	14.9	-	-
VviMET1	XM_002267164.3	LOC100251127	7	20.7	Rpv9	16.6
VviMET2 α	XM_002267248.4	LOC100240896	7	20.7	Rpv9	16.6

3.2. *Vitis vinifera* global 5-mC DNA levels modification upon *P. viticola* inoculation

The quantification of the 5-mC levels on genomic DNA was assessed in two different *Vitis vinifera* cultivars, 'Regent' (tolerant cultivar) and 'Trincadeira' (susceptible cultivar) at 6, 24, 48, 72 hours post-inoculation (hpi) and 5 days post-inoculation (dpi) with *P. viticola* (Figure 8). Later on, inoculated samples were compared to the non-inoculated controls.

Through the obtained results it was possible to verify that, in general, 'Trincadeira' presented higher levels of DNA methylation in the first and last timepoint after inoculation, when compared with 'Regent'. Furthermore, it is noteworthy that both cultivars reached their maximum level of methylation at 48 hpi.

The susceptible cultivar ‘Trincadeira’ presented a global hypermethylation pattern on the inoculated samples when compared to the non-inoculated controls. At 5 dpi, inoculated samples are hypomethylated. The tolerant cultivar ‘Regent’ presents an hypomethylation of the inoculated samples at 6 hpi, with a continuous increase in 5-mC methylation levels up to 48 hpi, where the higher methylation levels occur. At 5dpi, inoculated samples are again hypomethylated. It is noteworthy that only at 6 hpi, as shown in Figure 8, the difference between these two cultivars is statistically significant.

Leonetti and co-workers reported the presence of DNA methylation in resistant and susceptible tomato to root-knot nematodes. They showed that DNA hypomethylation was observed in roots from resistant tomato, while DNA hypermethylation was observed in successfully infected susceptible plants (Leonetti & Molinari 2020). Comparing with our results, we observed the same tendency. They also reported that the regulation in the incompatible interaction was associated with DNA hypomethylation, which could probably be linked with the inhibition of *de novo* methylation and the repression of methylation maintenance. Also, it was said that all DNA MTases tested in fully infected roots were up-regulated, sustaining the finding of a high DNA methylation state in such type of interactions with a promising consequent gene silencing (Leonetti & Molinari, 2020).

In 2019, Sun and his colleagues investigated the variation of global DNA methylation in watermelon (*Citrullus lanatus*) leaves after infection with the cucumber green mottle mosaic virus (CGMMV) at 0, 48 hpi, and 25 dpi (Sun *et al.*, 2019). In this study, the methylation levels varied greatly after CGMMV infection. The highest methylation level was observed in the control sample at 0 hpi and the lowest methylation level at 48 hpi. After that, the global methylation level increased in the 25 dpi (Sun *et al.*, 2019).

Hypomethylated genes were shown to present higher expression levels, while those that suffer hypermethylation were mainly shown to be down-regulated in plant-pathogen interactions (Hewezi *et al.*, 2017; Wang *et al.*, 2018; Geng *et al.*, 2019; Leonetti & Molinari, 2020). During cucumber mosaic virus infection on *N. benthamiana* tissues, differential methylated regions (DMRs) were positively correlated with gene expression, indicating the complex regulation of DNA methylation in the response of tobacco to viral processes (Wang *et al.*, 2018). Geng and co-workers also stated that, at least some, if not all, of the defense-related genes from *Aegilops tauschii* that were up-regulated upon *Blumeria graminis f.* sp. *tritici* (Bgt) infection, caused DNA hypomethylation, especially CHH hypomethylation, via TEs (Geng *et al.*, 2019).

Hypomethylation allows the transcription machinery to access the DNA more easily, enabling higher levels of gene transcription, including defense-related ones. Previous studies revealed that, during the time course of infection, signaling and defense mechanisms were preferentially more activated in ‘Regent’ (Figueiredo *et al*., 2012). The shown hypomethylation at 6 hpi may be contributing for the activation of gene transcription, which may in turn aid the plant to respond faster to the pathogen attack. The opposite can be observed when there is hypermethylation, where it can be more difficult to activate defense genes, since there is a greater methylation of DNA. Consequently, there will be a greater susceptibility to the pathogen, as is the case of the ‘Trincadeira’ cultivar.

At 5 dpi, hypomethylation is observed in both cultivars perhaps due to the absence of the stress condition at this timepoint.

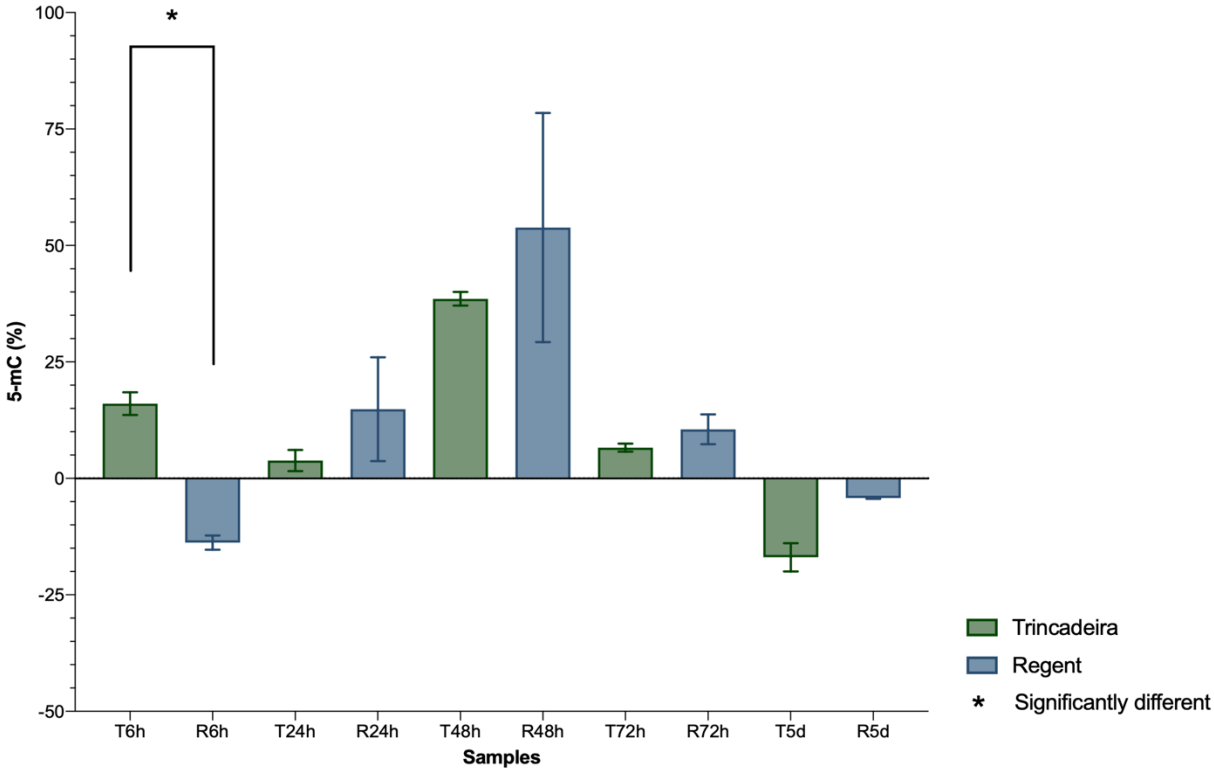


Figure 8 – Global 5-methylcytosine (5-mC) in *Vitis vinifera* cultivars – 'Trincadeira' (susceptible) and 'Regent' (tolerant) - at five different time points (6, 24, 48, 72 hpi, and 5 dpi), when compared to the non-inoculated controls. Asterisks (*) represent a significant difference (*p value* ≤ 0.05) between susceptible and tolerant cultivars.

3.3. Expression of grapevine DNA MTases genes involved in DNA methylation

In the present study, the expression profile of grapevine DNA methyltransferases (MTases) was evaluated in two *Vitis vinifera* cultivars, 'Regent' and 'Trincadeira', at five time-points 6, 24, 48, 72 hours post-inoculation (hpi) and 5 days post-inoculation (dpi). Melting curves of reference and targeted genes are shown in the Appendix 2 (Supplementary data 1).

When comparing the mock with the inoculated samples, the expression of some DNA methyltransferase genes was significantly altered in the later time-points on both genotypes. *VviDNMT1* presented a statistically significant modulation in 'Regent' at 6 hpi and 48 hpi; *VviDRM1* in 'Trincadeira' at 72 hpi and in 'Regent' at 6 and 48 hpi; *VviCMT2 α* in 'Trincadeira' at 6 hpi and in 'Regent' at 48 hpi and 5 dpi; *VviCMT1 α* at 6 hpi in 'Trincadeira' and at 24 hpi and 5 dpi in 'Regent'; and finally, *VviMET2 α* at 24 and 48 hpi in the 'Regent' cultivar (see Figure 9). DNA MTases expression cannot be directly associated to the global methylation patterns as our global analysis comprehends all different contexts of 5-mC and the genes studied code for DNA methyltransferases that promote sequence specific methylation. Nonetheless, their regulation presents some cues on the regulation of this process during grapevine - downy mildew interaction.

3.3.1. VviDNMT1

Studies focusing on methylation have not yet unraveled the function of this methyltransferase.
In 'Trincadeira', *VviDNMT1* expression was observed to be up-regulated at 6 and 24 hpi, down-regulated at 48 hpi and 72 hpi and then, at 5 dpi, the expression levels rose again. In contrast, expression patterns of this DNA MTase in the 'Regent' cultivar, exhibited a down-regulation at 6 hpi (*p value* ≤ 0.05), followed by a slight increase in expression at 24 hpi and becoming down-regulated again at 48 hpi. An increase of expression was then observed at 72 hpi and 5 dpi.
Overall, the expression of this MTase in the tolerant cultivar was characterized by a very pronounced down-regulation at 6 hpi and 48 hpi. Despite no direct relation can be made, in 'Regent' an hypomethylation pattern occurred at 6 hpi, thus at this specific time point the down-regulation of DNMT1 might be contributing for the methylation pattern identified.
Unfortunately, no studies have been found regarding the expression of this MTase gene in plant-pathogen interactions.

3.3.2. *VviDRM* genes

The *DRM* genes are known to catalyze *de novo* methylation (Leonetti & Molinari, 2020). Also, the maintenance of the CHH methylation includes the RdDM pathway and occurs *de novo* through the activity of DRM2 (Hewezi *et al.*, 2018; Leonetti & Molinari, 2020).
At 6 hpi, both V*viDRM1* and *VviDRM2* were up-regulated or presented no expression alteration in each cultivars (Figure 9). During the remaining inoculation time-course, in the compatible interaction (*V. vinifera* cv. 'Trincadeira'), both genes were mainly up-regulated while in the incompatible interaction (*V. vinifera* cv. 'Regent') there's a down-regulation at 48 hpi and 5 dpi.
In fact, a study on tomato roots inoculated with the root-knot nematode detected a down-regulation of *DRM* genes in the incompatible interaction (Leonetti & Molinari, 2020). The authors have hypothesized that DNA hypomethylation in the incompatible interaction can be associated with the suppression of de novo methylation and the inhibition of methylation maintenance (Leonetti & Molinari, 2020). A similar pattern is observed in grapevine compatible and incompatible interaction with *P. viticola* (Figure 8 and 9).

3.3.3. *VviCMT* genes

The CMT's primary function is to maintain methylation in CHG contexts through a reinforcing loop between CHG methylation and the methylation of lysine 9 of histone H3 (H3K9) (Zhu *et al.*, 2020). CHH methylation can also be performed by this class of MTases (Leonetti & Molinari, 2020).
In grapevine we have identified two clades, *VviCMT1* (similar to CMT1/CMT3/ZmMET in Angiosperms) and *VviCMT2*.

Considering both compatible and incompatible interactions, *VviCMT2 α* was significantly up-regulated in 'Trincadeira' at 6 hpi, remaining mostly up-regulated during the inoculation time-course. In 'Regent', this gene was down-regulated (see Figure 9) up to 5 dpi where it sustained a significant up-regulation. In the susceptible cultivar, the *VviCMT1 α* presented a statistically significant increase of expression at 6 hpi, while in the remaining inoculation time-course it presented a down-regulation at 48 and 72 hpi. *VviCMT1 β* was down-regulated at 24, 48 hpi and 5 dpi (Figure 9). In the tolerant cultivar 'Regent', both *VviCMT1 α* and *VviCMT1 β* presented the same modulation pattern, being down-regulated at most of the inoculation time-course with the exception of 24 hpi, where both genes were up-regulated (Figure 9). *VviCMT1* is similar to *CMT3,* previously shown to be involved in *de novo* DNA methylation (Kuźnicki *et al.*, 2019). *CMT3* expression was previously described to be accompanied by *ROS1* activation in a study where potato leaves were immunized by spraying with β-aminobutyric acid (BABA) (Kuźnicki *et al.*, 2019). The up-regulation of 'Regent' *VviCMT1* genes might be associated to the methylation pattern identified in 'Regent', with an increase of DNA methylation from 6 hpi to 48 hpi.

Moreover, *VviCMT* genes appear to be the most down-regulated in 'Regent', suggesting that their regulation might be associated with the DNA demethylation pattern. The down-regulation of the *CMT* genes has been previously reported in tomato roots inoculated with the root-knot nematode at 7 dpi (Leonetti & Molinari, 2020) and in watermelon when infected with cucumber green mottle mosaic virus at 48 hpi (Sun *et al*., 2019), further illustrating that this family of enzymes can be relevant in the context of infection.

3.3.4. *VviMET* genes

The CG methylation is maintained by methyltransferase 1 (MET1). The expression profile of both *VviMET1* and *VviMET2 α* genes was mainly constant throughout the inoculation time-course in both genotypes, except for *VviMET2 α* in 'Regent' that presented a statistically significant up-regulation at 24 hpi and a down-regulation at 48 hpi.

In the compatible interaction, *VviMET2 α* showed a similar up-regulation to that observed in tomato MET1 genes in roots inoculated with the root-knot nematodes (Leonetti & Molinari, 2020). The authors also stated that this up-regulation may support a high DNA methylation state with a possible consequent gene silencing (Leonetti & Molinari, 2020). The up-regulation of *VviMET2 α* in the compatible interaction may indicate the existence of gene silencing after *P. viticola* inoculation which is consistent with previous studies that shown down-regulation of defense-related transcripts in 'Trincadeira' leaves inoculated with *P. viticola* (Figueiredo *et al.,* 2012; Figueiredo *et al.,* 2016).

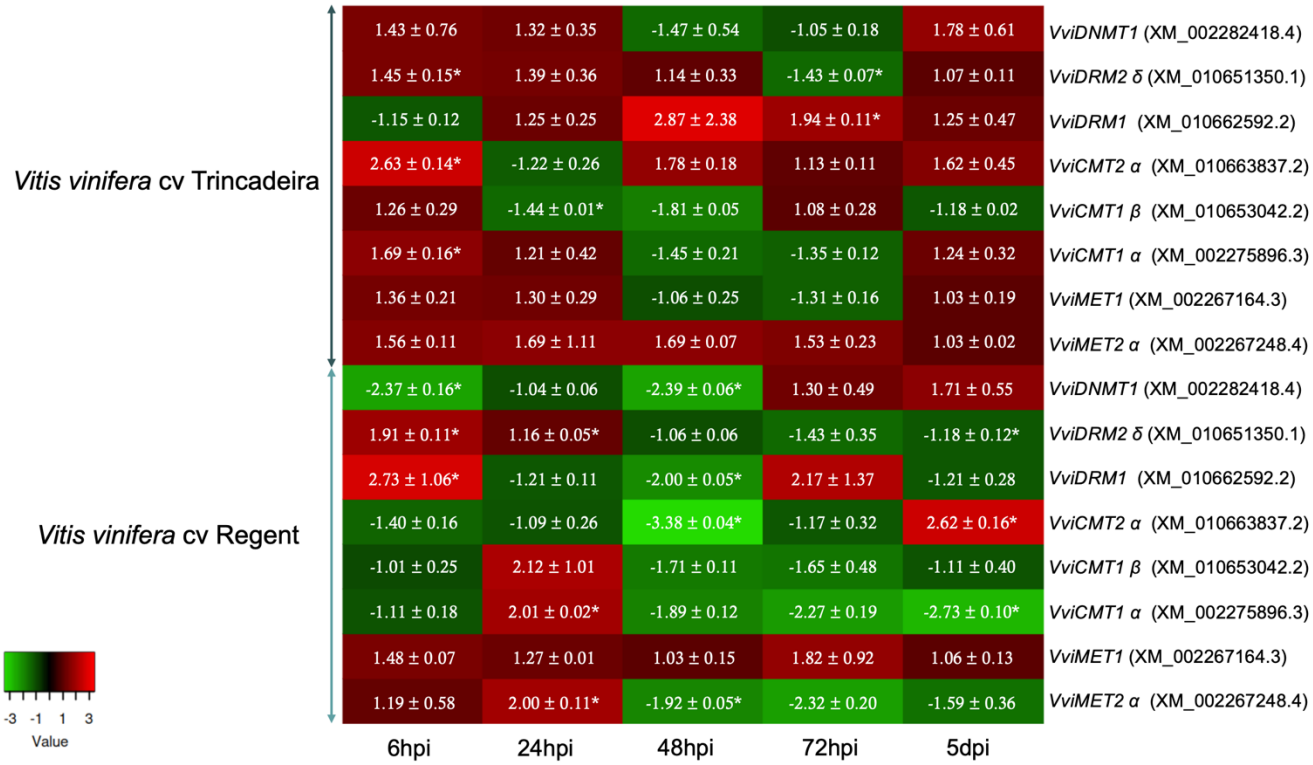


Figure 9 - Heatmap of 8 grapevine DNA C5-MTases expression in *V. vinifera* cv 'Regent' and *V. vinifera* cv 'Trincadeira' at 6, 24, 48, 72 hpi and 5 dpi with *P. viticola*. Each column indicates a time-point (6, 24, 48, 72 hpi and 5 dpi) and each row represents a MTase gene in the susceptible grapevine genotype ('Trincadeira') or in the tolerant grapevine genotype ('Regent'). Green indicates low expression levels, red indicates high expression levels. Asterisks (*) represents significant difference ($p \leq 0.05$) between target and control samples.

4. Conclusion and future perspectives

This project's main aim was to untangle the role of the *Vitis vinifera* DNA methyltransferase (MTase) gene family in the establishment of compatible and incompatible interactions between grapevine and *Plasmopara viticola*, the causal agent of downy mildew disease. Nine grapevine MTase genes were identified, distributed among 8 of the 19 grapevine chromosomes, coding for 17 methyltransferases proteins as a result of alternative splicing.

These MTases were grouped into four different classes, namely methyltransferase (MET), chromomethylases (CMT), domains rearranged methyltransferase (DRM), and DNA methyltransferase homolog (DNMT), according to their similarity. A subcellular location analysis showed that most of the grapevine MTases were located in the nucleus, vacuole, and chloroplast, which had been already described in the literature as some of the organelles with a significant role in the plant's stress responses. Moreover, some of the grapevine MTases genes showed to be located near to the loci associated with *Vitis vinifera* resistance against *Plasmopara viticola*.

The second goal of this project was to determine and correlate the DNA global methylation patterns with DNA MTase gene expression in two *Vitis vinifera* cultivars (‘Regent’ and ‘Trincadeira’) after *P. viticola* inoculation. A global analysis of 5-mC methylation and gene expression of grapevine DNA methyltransferases in the two different *Vitis vinifera* genotypes, susceptible and tolerant to *P. viticola,* was conducted. ‘Trincadeira’ 5-mC percentage results revealed that inoculated samples presented higher methylation than the control samples up to 5 dpi. Also, in ‘Trincadeira’, MTases genes were mostly up-regulated. On the other hand, ‘Regent’ gene expression analysis revealed that both the *CMT* genes and *DNMT1* appear to be the most down-regulated at both 6 and 48 hpi, suggesting that the down-regulation of these enzymes may be associated to the DNA demethylation pattern observed at 6 hpi and after 48 hpi. Our results may suggest that *CMT* and *DNMT1* genes may be involved in the epigenetic modulation associated to the establishment of the incompatible interaction.

In the future, it will be interesting to study, through bisulfite genomic sequencing analysis, the methylation state of the promoters of the defense genes in order to understand if there are differences between the two *V. vinifera* genotypes, ‘Regent’ and ‘Trincadeira’.

Furthermore, instead of performing global methylation analysis, it would also be exciting to study the concentration of each type of cytosine methylation along genic regions. It would be possible to see where there is a greater or lesser amount of methylation along the gene by using the bisulfite sequencing method. Just as several studies have done so far, by comparing with their expression, it would be possible to be more certain of the function of this gene.

Therefore, understanding the MTases family genes' methylation patterns in grapevine leaves would also be quite intriguing, as well as, studying the expression of DNA demethylation.

As can be concluded, epigenetics is a very dynamic mechanism with much to investigate and discover, both at the animal and plant level.

5. Bibliography

Ahmad, F., Huang, X., Lan, H. X., Huma, T., Bao, Y. M., Huang, J., & Zhang, H. S. (2014). Comprehensive gene expression analysis of the DNA (cytosine-5) methyltransferase family in rice (*Oryza sativa* L.). *Genetics and Molecular Research*, *13*(3), 5159–5172. https://doi.org/10.4238/2014.July.7.9

Ali, K., Maltese, F., Figueiredo, A., Rex, M., Fortes, A. M., Zyprian, E., Pais, M. S., Verpoorte, R., & Choi, Y. H. (2012). Alterations in grapevine leaf metabolism upon inoculation with *Plasmopara viticola* in different time-points. *Plant Science*, *191–192*, 100–107. https://doi.org/10.1016/j.plantsci.2012.04.014

Almagro Armenteros, J. J., Tsirigos, K. D., Sønderby, C. K., Petersen, T. N., Winther, O., Brunak, S., von Heijne, G., & Nielsen, H. (2019). SignalP 5.0 improves signal peptide predictions using deep neural networks. *Nature Biotechnology*, *37*(4), 420–423. https://doi.org/10.1038/s41587-019-0036-z

Armijo, G., Schlechter, R., Agurto, M., Muñoz, D., Nuñez, C., & Arce-Johnson, P. (2016). Grapevine pathogenic microorganisms: Understanding infection strategies and host response scenarios. In *Frontiers in Plant Science* (Vol. 7, Issue MAR2016, p. 382). Frontiers Media S.A. https://doi.org/10.3389/fpls.2016.00382

Ashapkin, V. V., Kutueva, L. I., & Vanyushin, B. F. (2016). Plant DNA methyltransferase genes: Multiplicity, expression, methylation patterns. *Biochemistry (Moscow)*, *81*(2), 141–151. https://doi.org/10.1134/S0006297916020085

Bej, S., & Basak, J. (2017). *Abiotic Stress Induced Epigenetic Modifications in Plants: How Much Do We Know?* 493–512. https://doi.org/10.1007/978-3-319-55520-1_24

Bewick, A. J., Niederhuth, C. E., Ji, L., Rohr, N. A., Griffin, P. T., Leebens-Mack, J., & Schmitz, R. J. (2017). The evolution of CHROMOMETHYLASES and gene body DNA methylation in plants. *Genome Biology*, *18*(1). https://doi.org/10.1186/s13059-017-1195-1

Bewick, A. J., & Schmitz, R. J. (2017). Gene body DNA methylation in plants. In *Current Opinion in Plant Biology* (Vol. 36, pp. 103–110). Elsevier Ltd. https://doi.org/10.1016/j.pbi.2016.12.007

Bochtler, M., Kolano, A., & Xu, G.-L. (2017). DNA demethylation pathways: Additional players and regulators. *BioEssays*, *39*(1), e201600178. https://doi.org/10.1002/bies.201600178

Brameier, M., Krings, A., & MacCallum, R. M. (2007). NucPred - Predicting nuclear localization of proteins. *Bioinformatics*, *23*(9), 1159–1160. https://doi.org/10.1093/bioinformatics/btm066

Brilli, M., Asquini, E., Moser, M., Bianchedi, P. L., Perazzolli, M., & Si-Ammour, A. (2018). A multi-omics study of the grapevine-downy mildew (*Plasmopara viticola*) pathosystem unveils a complex protein coding-A nd noncoding-based arms race during infection. *Scientific Reports*, *8*(1), 1–12. https://doi.org/10.1038/s41598-018-19158-8

Buonassisi, D., Colombo, M., Migliaro, D., Dolzani, C., Peressotti, E., Mizzotti, C., Velasco, R., Masiero, S., Perazzolli, M., & Vezzulli, S. (2017). Breeding for grapevine downy mildew resistance: a review of “omics” approaches. *Euphytica*, *213*(5). https://doi.org/10.1007/s10681-017-1882-8

Burruano, S. (2000). The life-cycle of *Plasmopara viticola*, cause of downy mildew of vine. *Mycologist*,

14(4), 179–182. https://doi.org/10.1016/S0269-915X(00)80040-3

Cao, D., Ju, Z., Gao, C., Mei, X., Fu, D., Zhu, H., Luo, Y., & Zhu, B. (2014). Genome-wide identification of cytosine-5 DNA methyltransferases and demethylases in *Solanum lycopersicum*. *Gene*, *550*(2), 230–237. https://doi.org/10.1016/j.gene.2014.08.034

Chang, L., Zhang, Z., Han, B., Li, H., Dai, H., He, P., & Tian, H. (2009). Isolation of dna-methyltransferase genes from strawberry (fragaria 3 ananassa duch.) and their expression in relation to micropropagation. *Plant Cell Reports*, *28*(9), 1373–1384. https://doi.org/10.1007/s00299-009-0737-8

Chen, W., Zhu, Q., Liu, Y., & Zhang, Q. (2017). Chromatin Remodeling and Plant Immunity. In *Advances in Protein Chemistry and Structural Biology* (Vol. 106, pp. 243–260). Academic Press Inc. https://doi.org/10.1016/bs.apcsb.2016.08.006

Chinnusamy, V., & Zhu, J. K. (2009). Epigenetic regulation of stress responses in plants. *Current Opinion in Plant Biology*, *12*(2), 133–139. https://doi.org/10.1016/j.pbi.2008.12.006

Ding, B., & Wang, G. L. (2015). Chromatin versus pathogens: The function of epigenetics in plant immunity. *Frontiers in Plant Science*, *6*(september), 1–8. https://doi.org/10.3389/fpls.2015.00675

Ding, X., Jimenez-Gongora, T., Krenz, B., & Lozano-Duran, R. (2019). Chloroplast clustering around the nucleus is a general response to pathogen perception in *Nicotiana benthamiana*. *Molecular Plant Pathology*, *20*(9), 1298–1306. https://doi.org/10.1111/mpp.12840

Divilov, K., Barba, P., Cadle-Davidson, L., & Reisch, B. I. (2018). Single and multiple phenotype QTL analyses of downy mildew resistance in interspecific grapevines. *Theoretical and Applied Genetics*, *131*(5), 1133–1143. https://doi.org/10.1007/s00122-018-3065-y

Dowen, R. H., Pelizzola, M., Schmitz, R. J., Lister, R., Dowen, J. M., Nery, J. R., Dixon, J. E., & Ecker, J. R. (2012). Widespread dynamic DNA methylation in response to biotic stress. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(32), E2183–E2191. https://doi.org/10.1073/pnas.1209329109

Du, J. (2016). Structure and mechanism of plant DNA methyltransferases. *Advances in Experimental Medicine and Biology*, *945*, 173–192. https://doi.org/10.1007/978-3-319-43624-1_8

EIP-AGRI. (2019). EIP-AGRI Focus Group Diseases and pests in viticulture. *Eip-Agri*, *March*, 1–66. http://ec.europa.eu/food/plant/pesticides/sustainable_use_pesticides/index_en.htm

Elhamamsy, A. R. (2016). DNA methylation dynamics in plants and mammals: overview of regulation and dysregulation. *Cell Biochemistry and Function*, *34*(5), 289–298. https://doi.org/10.1002/cbf.3183

Erdmann, R. M., & Picard, C. L. (2020). RNA-directed DNA Methylation. *PLoS Genetics*, *16*(10). https://doi.org/10.1371/journal.pgen.1009034

FAOSTAT. (n.d.). Retrieved March 16, 2020, from http://www.fao.org/faostat/en/#data/QC/visualize

Figueiredo, A., Fortes, A. M., Ferreira, S., Sebastiana, M., Choi, Y. H., Sousa, L., Acioli-Santos, B., Pessoa, F., Verpoorte, R., & Pais, M. S. (2008). Transcriptional and metabolic profiling of grape (*Vitis vinifera* L.) leaves unravel possible innate resistance against pathogenic fungi. *Journal of Experimental Botany*, *59*(12), 3371–3381. https://doi.org/10.1093/jxb/ern187

Figueiredo, A., Martins, J., Sebastiana, M., Guerreiro, A., Silva, A., Matos, A. R., Monteiro, F., Pais, M.

S., Roepstorff, P., & Coelho, A. V. (2017). Specific adjustments in grapevine leaf proteome discriminating resistant and susceptible grapevine genotypes to *Plasmopara viticola*. *Journal of Proteomics*, *152*, 48–57. https://doi.org/10.1016/j.jprot.2016.10.012

Figueiredo, A., Monteiro, F., Fortes, A. M., Bonow-Rex, M., Zyprian, E., Sousa, L., & Pais, M. S. (2012). Cultivar-specific kinetics of gene induction during downy mildew early infection in grapevine. *Functional and Integrative Genomics*, *12*(2), 379–386. https://doi.org/10.1007/s10142-012-0261-8

Finnegan, E. J., & Kovac, K. A. (2000). Plant DNA methyltransferases. *Plant Molecular Biology*, *43*(2–3), 189–201. https://doi.org/10.1023/a:1006427226972

Fortes, A. M., & Gallusci, P. (2017). Plant stress responses and phenotypic plasticity in the epigenomics era: Perspectives on the grapevine scenario, a model for perennial crop plants. *Frontiers in Plant Science*, *8*(FEBRUARY). https://doi.org/10.3389/fpls.2017.00082

Gahlaut, V., Samtani, H., & Khurana, P. (2020). Genome-wide identification and expression profiling of cytosine-5 DNA methyltransferases during drought and heat stress in wheat (*Triticum aestivum*). *Genomics*, *112*(6), 4796–4807. https://doi.org/10.1016/j.ygeno.2020.08.031

Gallego-Bartolomé, J. (2020). DNA methylation in plants: mechanisms and tools for targeted manipulation. In *New Phytologist* (Vol. 227, Issue 1, pp. 38–44). Blackwell Publishing Ltd. https://doi.org/10.1111/nph.16529

Geng, S., Kong, X., Song, G., Jia, M., Guan, J., Wang, F., Qin, Z., Wu, L., Lan, X., Li, A., & Mao, L. (2019). DNA methylation dynamics during the interaction of wheat progenitor *Aegilops tauschii* with the obligate biotrophic fungus *Blumeria graminis* f. sp. tritici. *New Phytologist*, *221*(2), 1023–1035. https://doi.org/10.1111/nph.15432

Gianoglio, S., Moglia, A., Acquadro, A., Comino, C., & Portis, E. (2017). The genome-wide identification and transcriptional levels of DNA methyltransferases and demethylases in globe artichoke. *PLOS ONE*, *12*(7), e0181669. https://doi.org/10.1371/journal.pone.0181669

Gindro, K., Spring, J. L., Pezet, R., Richter, H., & Viret, O. (2006). Histological and biochemical criteria for objective and early selection of grapevine cultivars resistant to *Plasmopara viticola*. *Vitis - Journal of Grapevine Research*, *45*(4), 191–196.

Gómez-Díaz, E., Jordà, M., Peinado, M. A., & Rivero, A. (2012). Epigenetics of Host-Pathogen Interactions: The Road Ahead and the Road Behind. *PLoS Pathogens*, *8*(11). https://doi.org/10.1371/journal.ppat.1003007

Gouveia, B. C., Calil, I. P., Machado, J. P. B., Santos, A. A., & Fontes, E. P. B. (2017). Immune receptors and co-receptors in antiviral innate immunity in plants. In *Frontiers in Microbiology* (Vol. 7, Issue JAN, p. 2139). Frontiers Media S.A. https://doi.org/10.3389/fmicb.2016.02139

He, C., Zhang, Z., Li, B., & Tian, S. (2020). The pattern and function of DNA methylation in fungal plant pathogens. In *Microorganisms* (Vol. 8, Issue 2). MDPI AG. https://doi.org/10.3390/microorganisms8020227

He, S., Xu, W., Li, F., Wang, Y., & Liu, A. (2017). Intraspecific DNA methylation polymorphism in the non-edible oilseed plant castor bean. *Plant Diversity*, *39*(5), 300–307. https://doi.org/10.1016/j.pld.2017.05.007

Hellemans, J., Mortier, G., De Paepe, A., Speleman, F., & Vandesompele, J. (2008). qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. *Genome Biology*, *8*(2), R19. https://doi.org/10.1186/gb-2007-8-2-r19

Hewezi, T., Lane, T., Piya, S., Rambani, A., Rice, J. H., & Staton, M. (2017). Cyst nematode parasitism induces dynamic changes in the root epigenome. *Plant Physiology*, *174*(1), 405–420. https://doi.org/10.1104/pp.16.01948

Hewezi, T., Pantalone, V., Bennett, M., Neal Stewart, C., & Burch-Smith, T. M. (2018). Phytopathogen-induced changes to plant methylomes. *Plant Cell Reports*, *37*(1), 17–23. https://doi.org/10.1007/s00299-017-2188-y

Hoang, T. V., Vo, K. T. X., Hong, W. J., Jung, K. H., & Jeon, J. S. (2018). Defense Response to Pathogens Through Epigenetic Regulation in Rice. In *Journal of Plant Biology* (Vol. 61, Issue 1). Springer New York LLC. https://doi.org/10.1007/s12374-017-0434-z

Islam, W., Noman, A., Qasim, M., & Wang, L. (2018). Plant responses to pathogen attack: Small rnas in focus. In *International Journal of Molecular Sciences* (Vol. 19, Issue 2). MDPI AG. https://doi.org/10.3390/ijms19020515

Jeltsch, A., & Jurkowska, R. Z. (2010). DNA Methylation. *The Chemical Biology of Nucleic Acids*, 307–321. https://doi.org/10.1002/9780470664001.ch14

Jones, J. D. G., & Dangl, J. L. (2006). The plant immune system. In *Nature* (Vol. 444, Issue 7117, pp. 323–329). https://doi.org/10.1038/nature05286

Jürges, G., Kassemeyer, H. H., Dürrenberger, M., Düggelin, M., & Nick, P. (2009). The mode of interaction between Vitis and *Plasmopara viticola* Berk. & Curt. Ex de Bary depends on the host species. *Plant Biology*, *11*(6), 886–898. https://doi.org/10.1111/j.1438-8677.2008.00182.x

Katoh, K., & Standley, D. M. (2013). MAFFT multiple sequence alignment software version 7: Improvements in performance and usability. *Molecular Biology and Evolution*, *30*(4), 772–780. https://doi.org/10.1093/molbev/mst010

Kelloniemi, J., Trouvelot, S., Héloir, M. C., Simon, A., Dalmais, B., Frettinger, P., Cimerman, A., Fermaud, M., Roudet, J., Baulande, S., Bruel, C., Choquer, M., Couvelard, L., Duthieuw, M., Ferrarini, A., Flors, V., Le Pêcheur, P., Loisel, E., Morgant, G., … Viaud, M. (2015). Analysis of the molecular dialogue between gray mold (*Botrytis cinerea*) and grapevine (*Vitis vinifera*) reveals a clear shift in defense mechanisms during berry ripening. *Molecular Plant-Microbe Interactions*, *28*(11), 1167–1180. https://doi.org/10.1094/MPMI-02-15-0039-R

Kiselev, K. V., Tyunin, A. P., & Karetin, Y. A. (2015). Salicylic acid induces alterations in the methylation pattern of the VaSTS1, VaSTS2, and VaSTS10 genes in *Vitis amurensis* Rupr. cell cultures. *Plant Cell Reports*, *34*(2), 311–320. https://doi.org/10.1007/s00299-014-1708-2

Kumar, S., Stecher, G., Li, M., Knyaz, C., & Tamura, K. (2018). MEGA X: Molecular evolutionary genetics analysis across computing platforms. *Molecular Biology and Evolution*, *35*(6), 1547–1549. https://doi.org/10.1093/molbev/msy096

Kuźnicki, D., Meller, B., Arasimowicz-Jelonek, M., Braszewska-Zalewska, A., Drozda, A., & Floryszak-Wieczorek, J. (2019). BABA-induced DNA methylome adjustment to intergenerational defense priming in potato to *Phytophthora infestans*. *Frontiers in Plant Science*, *10*, 650.

https://doi.org/10.3389/fpls.2019.00650

la Cour, T., Kiemer, L., Mølgaard, A., Gupta, R., Skriver, K., & Brunak, S. (2004). Analysis and prediction of leucine-rich nuclear export signals. *Protein Engineering, Design and Selection*, *17*(6), 527–536. https://doi.org/10.1093/protein/gzh062

Laloum, T., Martín, G., & Duque, P. (2018). Alternative Splicing Control of Abiotic Stress Responses. In *Trends in Plant Science* (Vol. 23, Issue 2, pp. 140–150). Elsevier Ltd. https://doi.org/10.1016/j.tplants.2017.09.019

Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., Mcgettigan, P. A., McWilliam, H., Valentin, F., Wallace, I. M., Wilm, A., Lopez, R., Thompson, J. D., Gibson, T. J., & Higgins, D. G. (2007). Clustal W and Clustal X version 2.0. *Bioinformatics*, *23*(21), 2947–2948. https://doi.org/10.1093/bioinformatics/btm404

Latouche, G., Bellow, S., Poutaraud, A., Meyer, S., & Cerovic, Z. G. (2013). Influence of constitutive phenolic compounds on the response of grapevine (*Vitis vinifera* L.) leaves to infection by *Plasmopara viticola*. *Planta*, *237*(1), 351–361. https://doi.org/10.1007/s00425-012-1776-x

Laureano, G., Figueiredo, J., Cavaco, A. R., Duarte, B., Caçador, I., Malhó, R., Sousa Silva, M., Matos, A. R., & Figueiredo, A. (2018). The interplay between membrane lipids and phospholipase A family members in grapevine resistance against *Plasmopara viticola*. *Scientific Reports*, *8*(1), 1–15. https://doi.org/10.1038/s41598-018-32559-z

Law, J. A., & Jacobsen, S. E. (2010). Establishing, maintaining and modifying DNA methylation patterns in plants and animals. In *Nature Reviews Genetics* (Vol. 11, Issue 3, pp. 204–220). https://doi.org/10.1038/nrg2719

Le, T. N., Schumann, U., Smith, N. A., Tiwari, S., Khang Au, P. C., Zhu, Q. H., Taylor, J. M., Kazan, K., Llewellyn, D. J., Zhang, R., Dennis, E. S., & Wang, M. B. (2014). DNA demethylases target promoter transposable elements to positively regulate stress responsive genes in *Arabidopsis*. *Genome Biology*, *15*(9), 1–18. https://doi.org/10.1186/s13059-014-0458-3

Leonetti, P., & Molinari, S. (2020). Epigenetic and Metabolic Changes in Root-Knot Nematode-Plant Interactions. *International Journal of Molecular Sciences*, *21*(20), 7759. https://doi.org/10.3390/ijms21207759

Li, J., Li, C., & Lu, S. (2018). Identification and characterization of the cytosine-5 DNA methyltransferase gene family in *Salvia miltiorrhiza*. *PeerJ*, *2018*(3). https://doi.org/10.7717/peerj.4461

Li, Q., Eichten, S. R., Hermanson, P. J., Zaunbrecher, V. M., Song, J., Wendt, J., Rosenbaum, H., Madzima, T. F., Sloan, A. E., Huang, J., Burgess, D. L., Richmond, T. A., McGinnis, K. M., Meeley, R. B., Danilevskaya, O. N., Vaughn, M. W., Kaeppler, S. M., Jeddeloh, J. A., & Springer, N. M. (2014). Genetic perturbation of the maize methylome. *Plant Cell*, *26*(12), 4602–4616. https://doi.org/10.1105/tpc.114.133140

Li, Y., Kumar, S., & Qian, W. (2018). Active DNA demethylation: mechanism and role in plant development. In *Plant Cell Reports* (Vol. 37, Issue 1, pp. 77–85). Springer Verlag. https://doi.org/10.1007/s00299-017-2215-z

Liu, Ruie, & Lang, Z. (2020). The mechanism and function of active DNA demethylation in plants. *Journal of Integrative Plant Biology*, *62*(1), 148–159. https://doi.org/10.1111/jipb.12879

Liu, Ruiqi, Wang, L., Zhu, J., Chen, T., Wang, Y., & Xu, Y. (2014). Histological responses to downy mildew in resistant and susceptible grapevines. *Protoplasma*, *252*(1), 259–270. https://doi.org/10.1007/s00709-014-0677-1

Malik, G., Dangwal, M., Kapoor, S., & Kapoor, M. (2012). Role of DNA methylation in growth and differentiation in *Physcomitrella patens* and characterization of cytosine DNA methyltransferases. *FEBS Journal*, *279*(21), 4081–4094. https://doi.org/10.1111/febs.12002

Méndez, K. Á., & Romero, H. M. (2017). Plant responses to pathogen attack: Molecular basis of qualitative resistance. In *Revista Facultad Nacional de Agronomia Medellin* (Vol. 70, Issue 2, pp. 8225–8235). Universidad Nacional de Colombia. https://doi.org/10.15446/rfna.v70n2.64526

Merz, P. R., Moser, T., Höll, J., Kortekamp, A., Buchholz, G., Zyprian, E., & Bogs, J. (2015). The transcription factor VvWRKY33 is involved in the regulation of grapevine (*Vitis vinifera*) defense against the oomycete pathogen *Plasmopara viticola*. *Physiologia Plantarum*, *153*(3), 365–380. https://doi.org/10.1111/ppl.12251

Milli, A., Cecconi, D., Bortesi, L., Persi, A., Rinalducci, S., Zamboni, A., Zoccatelli, G., Lovato, A., Zolla, L., & Polverari, A. (2012). Proteomic analysis of the compatible interaction between *Vitis vinifera* and *Plasmopara viticola*. *Journal of Proteomics*, *75*(4), 1284–1302. https://doi.org/10.1016/j.jprot.2011.11.006

Moglia, A., Gianoglio, S., Acquadro, A., Valentino, D., Milani, A. M., Lanteri, S., & Comino, C. (2019). Identification of DNA methyltransferases and demethylases in *Solanum melongena* L., and their transcription dynamics during fruit development and after salt and drought stresses. *PLOS ONE*, *14*(10), e0223581. https://doi.org/10.1371/journal.pone.0223581

Monteiro, F., Sebastiana, M., Pais, M. S., & Figueiredo, A. (2013). Reference Gene Selection and Validation for the Early Responses to Downy Mildew Infection in Susceptible and Resistant *Vitis vinifera* Cultivars. *PLoS ONE*, *8*(9), e72998. https://doi.org/10.1371/journal.pone.0072998

Negrel, L., Halter, D., Wiedemann-Merdinoglu, S., Rustenholz, C., Merdinoglu, D., Hugueney, P., & Baltenweck, R. (2018). Identification of lipid markers of *Plasmopara viticola* infection in grapevine using a non-targeted metabolomic approach. *Frontiers in Plant Science*, *9*. https://doi.org/10.3389/fpls.2018.00360

OIV. (2019). 2019 Statistical Report on World Vitiviniculture. In *The International Organisation of Vine and Wine*. https://doi.org/64/19/6835 [pii]\n10.1158/0008-5472.CAN-04-1678

OIV. (2020). *2020 WINE PRODUCTION OIV First Estimates*.

Pavlopoulou, A., & Kossida, S. (2007). Plant cytosine-5 DNA methyltransferases: Structure, function, and molecular evolution. *Genomics*, *90*(4), 530–541. https://doi.org/10.1016/j.ygeno.2007.06.011

Pikaard, C. S., & Scheid, O. M. (2014). Epigenetic regulation in plants. *Cold Spring Harbor Perspectives in Biology*, *6*(12). https://doi.org/10.1101/cshperspect.a019315

Polesani, M., Desario, F., Ferrarini, A., Zamboni, A., Pezzotti, M., Kortekamp, A., & Polverari, A. (2008). cDNA-AFLP analysis of plant and pathogen genes expressed in grapevine infected with *Plasmopara viticola*. *BMC Genomics*, *9*(1), 142. https://doi.org/10.1186/1471-2164-9-142

Ponzio, C., Weldegergis, B. T., Dicke, M., & Gols, R. (2016). Compatible and incompatible pathogen–plant interactions differentially affect plant volatile emissions and the attraction of parasitoid wasps.

Functional Ecology, *30*(11), 1779–1789. https://doi.org/10.1111/1365-2435.12689

Possamai, T., Migliaro, D., Gardiman, M., Velasco, R., & De Nardi, B. (2020). Rpv mediated defense responses in grapevine offspring resistant to plasmopara viticola. *Plants*, *9*(6), 1–10. https://doi.org/10.3390/plants9060781

Qian, Y., Xi, Y., Cheng, B., & Zhu, S. (2014). Genome-wide identification and expression profiling of DNA methyltransferase gene family in maize. *Plant Cell Reports*, *33*(10), 1661–1672. https://doi.org/10.1007/s00299-014-1645-0

Qiu, W., Feechan, A., & Dry, I. (2015). Current understanding of grapevine defense mechanisms against the biotrophic fungus (*Erysiphe necator*), the causal agent of powdery mildew disease. In *Horticulture Research* (Vol. 2, Issue 1, pp. 1–9). Nature Publishing Group. https://doi.org/10.1038/hortres.2015.20

Rambani, A., Pantalone, V., Yang, S., Rice, J. H., Song, Q., Mazarei, M., Arelli, P. R., Meksem, K., Stewart, C. N., & Hewezi, T. (2020). Identification of introduced and stably inherited DNA methylation variants in soybean associated with soybean cyst nematode parasitism. *New Phytologist*, *227*(1), 168–184. https://doi.org/10.1111/nph.16511

Ramirez-Prado, J. S., Piquerez, S. J. M., Bendahmane, A., Hirt, H., Raynaud, C., & Benhamed, M. (2018). Modify the histone to win the battle: Chromatin dynamics in plant–pathogen interactions. In *Frontiers in Plant Science* (Vol. 9). Frontiers Media S.A. https://doi.org/10.3389/fpls.2018.00355

Sahu, P. P., Pandey, G., Sharma, N., Puranik, S., Muthamilarasan, M., & Prasad, M. (2013). Epigenetic mechanisms of plant stress responses and adaptation. In *Plant Cell Reports* (Vol. 32, Issue 8, pp. 1151–1159). Springer. https://doi.org/10.1007/s00299-013-1462-x

Santos, R. B., & Figueiredo, A. (2021). Two sides of the same story in grapevine-pathogen interactions. *Journal of Experimental Botany*. https://doi.org/10.1093/jxb/erab091

Santos, R. B., Nascimento, R., Coelho, A. V., & Figueiredo, A. (2020). Grapevine–downy mildew Rendezvous: Proteome analysis of the first hours of an incompatible interaction. *Plants*, *9*(11), 1–17. https://doi.org/10.3390/plants9111498

Schübeler, D. (2015). Function and information content of DNA methylation. *Nature*, *517*(7534), 321–326. https://doi.org/10.1038/nature14192

Seffer, I., Nemeth, Z., Hoffmann, G., Matics, R., Seffer, A. G., & Koller, A. (2013). Unexplored Potentials of Epigenetic Mechanisms of Plants and Animals—Theoretical Considerations. *Genetics & Epigenetics*, *5*, GEG.S11752. https://doi.org/10.4137/geg.s11752

Sharma, R., Mohan Singh, R. K., Malik, G., Deveshwar, P., Tyagi, A. K., Kapoor, S., & Kapoor, M. (2009). Rice cytosine DNA methyltransferases - gene expression profiling during reproductive development and abiotic stress. *FEBS Journal*, *276*(21), 6301–6311. https://doi.org/10.1111/j.1742-4658.2009.07338.x

Shen, H. Bin, & Chou, K. C. (2007). Nuc-PLoc: A new web-server for predicting protein subnuclear localization by fusing PseAA composition and PsePSSM. *Protein Engineering, Design and Selection*, *20*(11), 561–567. https://doi.org/10.1093/protein/gzm057

Sperschneider, J., Catanzariti, A. M., Deboer, K., Petre, B., Gardiner, D. M., Singh, K. B., Dodds, P. N., & Taylor, J. M. (2017). LOCALIZER: Subcellular localization prediction of both plant and effector

proteins in the plant cell. *Scientific Reports*, *7*. https://doi.org/10.1038/srep44598

Sudan, J., Raina, M., & Singh, R. (2018). Plant epigenetic mechanisms: role in abiotic stress and their generational heritability. In *3 Biotech* (Vol. 8, Issue 3, p. 172). Springer Verlag. https://doi.org/10.1007/s13205-018-1202-6

Sun, Y., Fan, M., & He, Y. (2019). DNA methylation analysis of the *Citrullus lanatus* response to *Cucumber Green Mottle Mosaic Virus* infection by whole-genome bisulfite sequencing. *Genes*, *10*(5). https://doi.org/10.3390/genes10050344

Taylor, A. S., Knaus, B. J., Grünwald, N. J., & Burgess, T. (2019). Population Genetic Structure and Cryptic Species of *Plasmopara viticola* in Australia. *Phytopathology*, *109*(11), 1975–1983. https://doi.org/10.1094/PHYTO-04-19-0146-R

Terral, J. F., Tabard, E., Bouby, L., Ivorra, S., Pastor, T., Figueiral, I., Picq, S., Chevance, J. B., Jung, C., Fabre, L., Tardy, C., Compan, M., Bacilieri, R., Lacombe, T., & This, P. (2010). Evolution and history of grapevine (*Vitis vinifera*) under domestication: new morphometric perspectives to understand seed domestication syndrome and reveal origins of ancient European cultivars. *Annals of Botany*, *105*(3), 443–455. https://doi.org/10.1093/aob/mcp298

Teyssier, E., Bernacchia, G., Maury, S., How Kit, A., Stammitti-Bert, L., Rolin, D., & Gallusci, P. (2008). Tissue dependent variations of DNA methylation and endoreduplication levels during tomato fruit development and ripening. *Planta*, *228*(3), 391–399. https://doi.org/10.1007/s00425-008-0743-z

Thatcher, S. R., Danilevskaya, O. N., Meng, X., Beatty, M., Zastrow-Hayes, G., Harris, C., Van Allen, B., Habben, J., & Li, B. (2016). Genome-wide analysis of alternative splicing during development and drought stress in maize1[OPEN]. *Plant Physiology*, *170*(1), 586–599. https://doi.org/10.1104/pp.15.01267

Tirnaz, S., & Batley, J. (2019a). DNA Methylation: Toward Crop Disease Resistance Improvement. *Trends in Plant Science*, *24*(12), 1137–1150. https://doi.org/10.1016/j.tplants.2019.08.007

Toffolatti, S. L., De Lorenzis, G., Costa, A., Maddalena, G., Passera, A., Bonza, M. C., Pindo, M., Stefani, E., Cestaro, A., Casati, P., Failla, O., Bianco, P. A., Maghradze, D., & Quaglino, F. (2018). Unique resistance traits against downy mildew from the center of origin of grapevine (*Vitis vinifera*). *Scientific Reports*, *8*(1), 1–11. https://doi.org/10.1038/s41598-018-30413-w

Vaissière, T., Sawan, C., & Herceg, Z. (2008). Epigenetic interplay between histone modifications and DNA methylation in gene silencing. *Mutation Research - Reviews in Mutation Research*, *659*(1–2), 40–48. https://doi.org/10.1016/j.mrrev.2008.02.004

van der Burgh, A. M., & Joosten, M. H. A. J. (2019). Plant Immunity: Thinking Outside and Inside the Box. In *Trends in Plant Science* (Vol. 24, Issue 7, pp. 587–601). Elsevier Ltd. https://doi.org/10.1016/j.tplants.2019.04.009

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., & Speleman, F. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology*, *3*(7), research0034.1. https://doi.org/10.1186/gb-2002-3-7-research0034

Vanyushin, B. F., & Ashapkin, V. V. (2011). DNA methylation in plants. *DNA Methylation in Plants*, 1–152. https://doi.org/10.1146/annurev.arplant.49.1.223

Veloso, M. M., Almandanim, M. C., Baleiras-Couto, M., Pereira, H., Carneiro, L. C., Fevereiro, P., & Eiras-Dias, J. (2010). *MICROSATELLITE DATABASE OF GRAPEVINE (Vitis vinifera L.) CULTIVARS USED FOR WINE PRODUCTION IN PORTUGAL*. www.inrb.pt

Victoria, D., Aliki, K., Venetia, K., Georgios, M., & Zoe, H. (2018). Spatial and temporal expression of cytosine-5 DNA methyltransferase and DNA demethylase gene families of the *Ricinus communis* during seed development and drought stress. *Plant Growth Regulation*, *84*(1), 81–94. https://doi.org/10.1007/s10725-017-0323-y

Vitulo, N., Forcato, C., Carpinelli, E. C., Telatin, A., Campagna, D., D'Angelo, M., Zimbello, R., Corso, M., Vannozzi, A., Bonghi, C., Lucchin, M., & Valle, G. (2014). A deep survey of alternative splicing in grape reveals changes in the splicing machinery related to tissue, stress condition and genotype. *BMC Plant Biology*, *14*(1), 99. https://doi.org/10.1186/1471-2229-14-99

VIVC. (n.d.). Retrieved November 11, 2020, from https://www.vivc.de/

Wang, C., Wang, C., Xu, W., Zou, J., Qiu, Y., Kong, J., Yang, Y., Zhang, B., & Zhu, S. (2018). Epigenetic changes in the regulation of *Nicotiana tabacum* response to cucumber mosaic virus infection and symptom recovery through single-base resolution methylomes. *Viruses*, *10*(8). https://doi.org/10.3390/v10080402

Wang, P., Gao, C., Bian, X., Zhao, S., Zhao, C., Xia, H., Song, H., Hou, L., Wan, S., & Wang, X. (2016). Genome-wide identification and comparative analysis of cytosine-5 DNA methyltransferase and demethylase families in wild and cultivated peanut. *Frontiers in Plant Science*, *7*(FEB2016), 1–14. https://doi.org/10.3389/fpls.2016.00007

Weiberg, A., & Jin, H. (2015). Small RNAs-the secret agents in the plant-pathogen interactions. In *Current Opinion in Plant Biology* (Vol. 26, pp. 87–94). Elsevier Ltd. https://doi.org/10.1016/j.pbi.2015.05.033

Welter, L. J., Göktürk-Baydar, N., Akkurt, M., Maul, E., Eibach, R., Töpfer, R., & Zyprian, E. M. (2007). Genetic mapping and localization of quantitative trait loci affecting fungal disease resistance and leaf morphology in grapevine (*Vitis vinifera* L). *Molecular Breeding*, *20*(4), 359–374. https://doi.org/10.1007/s11032-007-9097-7

Wendte, J. M., & Schmitz, R. J. (2018). Specifications of Targeting Heterochromatin Modifications in Plants. In *Molecular Plant* (Vol. 11, Issue 3, pp. 381–387). Cell Press. https://doi.org/10.1016/j.molp.2017.10.002

WILLIAMSON, B., TUDZYNSKI, B., TUDZYNSKI, P., & VAN KAN, J. A. L. (2007). Botrytis cinerea: the cause of grey mould disease. *Molecular Plant Pathology*, *8*(5), 561–580. https://doi.org/10.1111/j.1364-3703.2007.00417.x

Wilson, C., & Tisdell, C. (2001). Why farmers continue to use pesticides despite environmental, health and sustainability costs. *Ecological Economics*, *39*(3), 449–462. https://doi.org/10.1016/S0921-8009(01)00238-5

Wu, C.-T., & Morris, J. R. (2001). *E P I G E N E T I C S s Genes, Genetics, and Epigenetics: A Correspondence*. *293*(August), 1103–1105. www.sciencernag.org

Wu, K. J. (2020). The epigenetic roles of DNA N6-Methyladenine (6mA) modification in eukaryotes. In *Cancer Letters* (Vol. 494, pp. 40–46). Elsevier Ireland Ltd.

https://doi.org/10.1016/j.canlet.2020.08.025

Yadav, D., Tanveer, A., Malviya, N., & Yadav, S. (2018). Overview and principles of bioengineering: The drivers of omics technologies. In *Omics Technologies and Bio-engineering: Towards Improving Quality of Life* (Vol. 1, pp. 3–23). Elsevier Inc. https://doi.org/10.1016/B978-0-12-804659-3.00001-4

Yamauchi, T., Moritoh, S., Johzuka-Hisatomi, Y., Ono, A., Terada, R., Nakamura, I., & Iida, S. (2008). Alternative splicing of the rice OsMET1 genes encoding maintenance DNA methyltransferase. *Journal of Plant Physiology*, *165*(17), 1774–1782. https://doi.org/10.1016/j.jplph.2007.12.003

Yin, L., An, Y., Qu, J., Li, X., Zhang, Y., Dry, I., Wu, H., & Lu, J. (2017). Genome sequence of Plasmopara viticola and insight into the pathogenic mechanism. *Scientific Reports*, *7*(1), 1–12. https://doi.org/10.1038/srep46553

Yu, A., Lepère, G., Jay, F., Wang, J., Bapaume, L., Wang, Y., Abraham, A. L., Penterman, J., Fischer, R. L., Voinnet, O., & Navarro, L. (2013). Dynamics and biological relevance of DNA demethylation in *Arabidopsis* antibacterial defense. *Proceedings of the National Academy of Sciences of the United States of America*, *110*(6), 2389–2394. https://doi.org/10.1073/pnas.1211757110

Yu, Y., Jiao, L., Fu, S., Yin, L., Zhang, Y., & Lu, J. (2016). Callose Synthase Family Genes Involved in the Grapevine Defense Response to Downy Mildew Disease. *Phytopathology®*, *106*(1), 56–64. https://doi.org/10.1094/PHYTO-07-15-0166-R

Zdunić, G., Preece, J. E., Aradhya, M., Velasco, D., Koehmstedt, A., & Dangl, G. S. (2013). Genetic diversity and differentiation within and between cultivated (*Vitis vinifera* L. ssp. sativa) and wild (*Vitis vinifera* L. ssp. sylvestris) grapes. *Vitis - Journal of Grapevine Research*, *52*(1), 29–32. https://www.researchgate.net/publication/284438048_Genetic_diversity_and_differentiation_within_and_between_cultivated_Vitis_vinifera_L_ssp_sativa_and_wild_Vitis_vinifera_L_ssp_sylvestris_grapes

Zhang, H., Lang, Z., & Zhu, J. K. (2018). Dynamics and function of DNA methylation in plants. *Nature Reviews Molecular Cell Biology*, *19*(8), 489–506. https://doi.org/10.1038/s41580-018-0016-z

Zhang, Y., He, X., Zhao, H., Xu, W., Deng, H., Wang, H., Wang, S., Su, D., Zheng, Z., Yang, B., Grierson, D., Wu, J., & Liu, M. (2020). Genome-Wide Identification of DNA Methylases and Demethylases in Kiwifruit (*Actinidia chinensis*). *Frontiers in Plant Science*, *11*, 1380. https://doi.org/10.3389/fpls.2020.514993

Zhu, C., Zhang, S., Zhou, C., Chen, L., Fu, H., Li, X., Lin, Y., Lai, Z., & Guo, Y. (2020). Genome-wide investigation and transcriptional analysis of cytosine-5 DNA methyltransferase and DNA demethylase gene families in tea plant (*Camellia sinensis*) under abiotic stress and withering processing. *PeerJ*, *2020*(1). https://doi.org/10.7717/peerj.8432

6. Appendix

6.1. Appendix 1

Supplementary table 1 - General features of grapevine DNA methyltransferases. Adopted identifier, gene, nucleotide and protein accessions, protein name predicted, enzymes code, length, molecular weight, and isoelectric point are represented.

	NCBI									
Adopted identifier	**Chr**	**Gene Locus**	**Start (bp)**	**End (bp)**	**Position (mean)**	**Nucleotide**	**Protein**	**Sequence Description (Predicted)**	**Length (aa)**	**Mol Wt. (kDa)**	**pI**
VviDNMT1	4	LOC109121395	4545263	4550254	4,545	XM_002282418.4	XP_002282454.2	tRNA (cytosine-5-)-methyltransferase	4992	44,85	5,77
VviDRM1	14	LOC100254189	27478355	27491506	27,478	XM_010662592.2	XP_010660894.1	DNA (cytosine-5)-methyltransferase DRM2	13152	67,63	4,93
VviDRM2 δ	5	LOC100247823	2360357	2376603	2,360	XM_010651350.1	XP_010649652.1	probable inactive DNA (cytosine-5)-methyltransferase DRM3 isoform X1	16247	82,37	5,62
VviDRM2 β	5	LOC100247823	2360357	2376603	2,360	XM_019220153.1	XP_019075698.1	probable inactive DNA (cytosine-5)-methyltransferase DRM3 isoform X2	16247	80,14	5,54
VviDRM2 γ	5	LOC100247823	2360357	2376603	2,360	XM_002264190.2	XP_002264226.1	probable inactive DNA (cytosine-5)-methyltransferase DRM3 isoform X3	16247	79,80	5,63
VviDRM2 ε	5	LOC100247823	2360357	2376603	2,360	XM_019220154.1	XP_019075699.1	probable inactive DNA (cytosine-5)-methyltransferase DRM3 isoform X4	16247	78,44	5,78
VviDRM2 α	5	LOC100247823	2360357	2376603	2,360	XM_019220155.1	XP_019075700.1	probable inactive DNA (cytosine-5)-methyltransferase DRM3 isoform X5	16247	66,84	6,36
VviCMT1 α	8	LOC100252100	20455472	20461551	20,455	XM_002275896.3	XP_002275932.1	putative DNA (cytosine-5)-methyltransferase CMT1	6080	92,74	5,64

Supplementary table 1 (cont.) - General features of grapevine DNA methyltransferases. Adopted identifier, gene, nucleotide and protein accessions, protein name predicted, enzymes code, length, molecular weight, and isoelectric point are represented.

NCBI											
Adopted identifier	**Chr**	**Gene Locus**	**Start (bp)**	**End (bp)**	**Position (mean)**	**Nucleotide**	**Protein**	**Sequence Description (Predicted)**	**Length (aa)**	**Mol Wt. (kDa)**	**pI**
VviCMT2 α	16	LOC100249359	2651859	2694284	2,652	XM_010663837.2	XP_010662139.1	DNA (cytosine-5)-methyltransferase CMT2 isoform X2	42426	100,35	8,37
VviCMT2 ε	2	LOC100255190	14901820	14984120	14,902	XM_010666729.2	XP_010665031.1	DNA (cytosine-5)-methyltransferase CMT2 isoform X1	82301	160,80	8,75
VviCMT2 δ	2	LOC100255190	14901820	14984120	14,902	XM_010666733.2	XP_010665035.1	DNA (cytosine-5)-methyltransferase CMT2 isoform X2	82301	160,55	8,75
VviCMT2 γ	2	LOC100255190	14901820	14984120	14,902	XM_010666739.2	XP_010665041.1	DNA (cytosine-5)-methyltransferase CMT2 isoform X4	82301	151,24	8,84
VviCMT2 β	2	LOC100255190	14901820	14984120	14,902	XM_019225253.1	XP_019080798.1	DNA (cytosine-5)-methyltransferase CMT2 isoform X3	82301	153,52	8,75
VviCMT1 β	6	LOC100258788	1170549	1184405	1,171	XM_010653042.2	XP_010651344.1	DNA (cytosine-5)-methyltransferase CMT3	13857	108,07	5,25
VviMET1	7	LOC100251127	20695405	20704884	20,695	XM_002267164.3	XP_002267200.1	DNA (cytosine-5)-methyltransferase 1	9480	174,74	5,79
VviMET2 α	7	LOC100240896	20683937	20691274	20,684	XM_002267248.4	XP_002267284.3	DNA (cytosine-5)-methyltransferase 1 isoform X1	7338	174,62	5,93
VviMET2 β	7	LOC100240896	20683937	20691274	20,684	XM_019221170.1	XP_019076715.1	DNA (cytosine-5)-methyltransferase 1 isoform X2	7338	176,94	6,02

Supplementary table 2 - Domain prediction results from the identified grapevine DNA methyltransferases

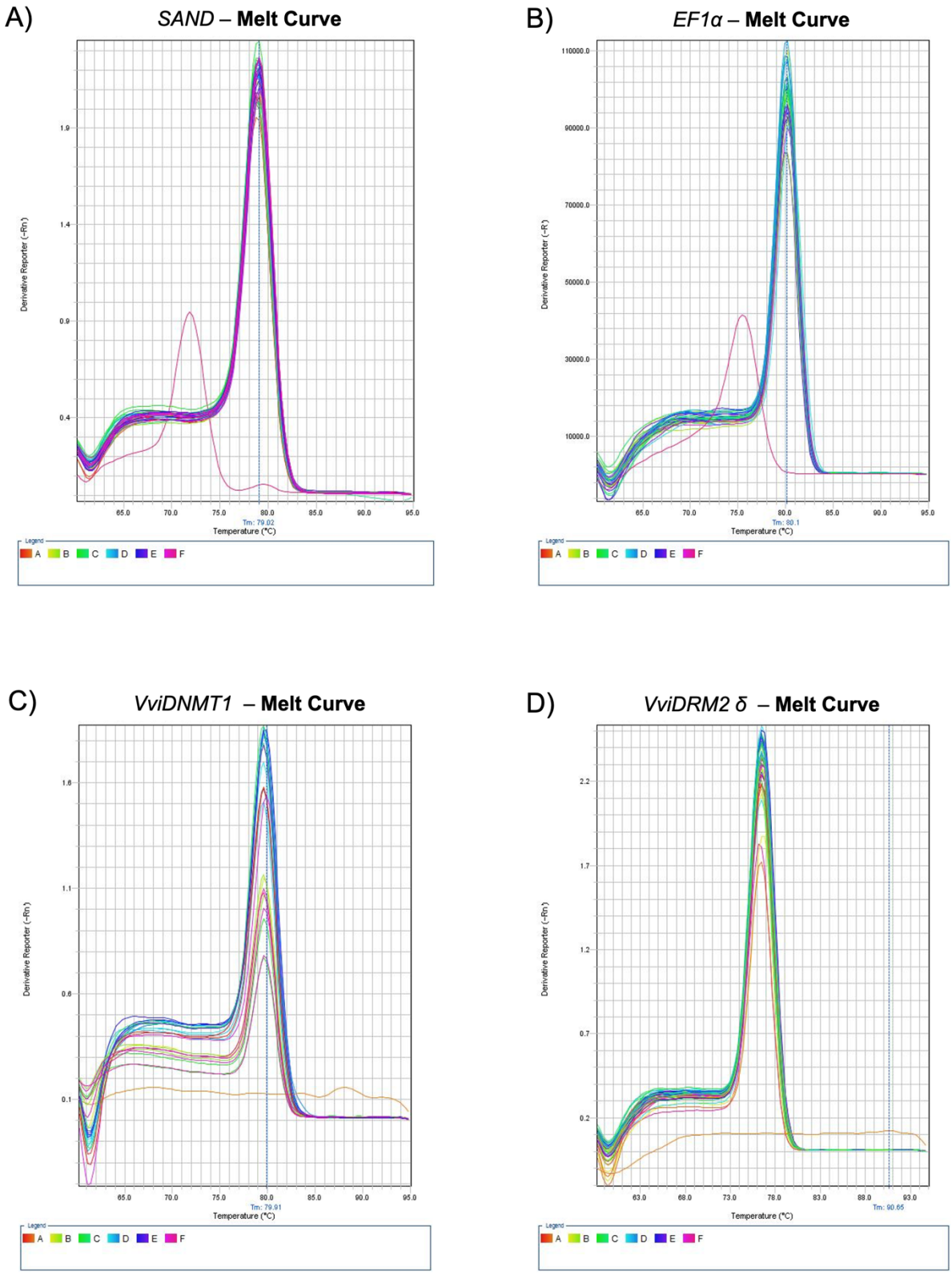
	Domains				
Adopted identifier	**DNMT1-RFD**	**BAH**	**Chromo**	**DCM**	**AdoMet_MTases**
VviDNMT1	0	0	0	0	1
VviDRM1	0	0	0	1	1
VviDRM2 δ	0	0	0	0	1
VviDRM2 β	0	0	0	0	0
VviDRM2 γ	0	0	0	0	1
VviDRM2 ε	0	0	0	0	0
VviDRM2 α	0	0	0	0	0
VviCMT1 α	0	1	0	1	0
VviCMT2 α	0	1	1	1	0
VviCMT2 ε	0	1	1	1	0
VviCMT2 δ	0	1	1	1	0
VviCMT2 γ	0	1	0	1	0
VviCMT2 β	0	0	0	0	0
VviCMT1 β	0	1	1	1	0
VviMET1	2	2	0	1	0
VviMET2 α	2	2	0	1	0
VviMET2 β	0	0	0	0	0

Supplementary table 3 - Gene structure analysis of grapevine methyltransferases. Open Reading Frame (ORF) length and the number of exons and introns are represented

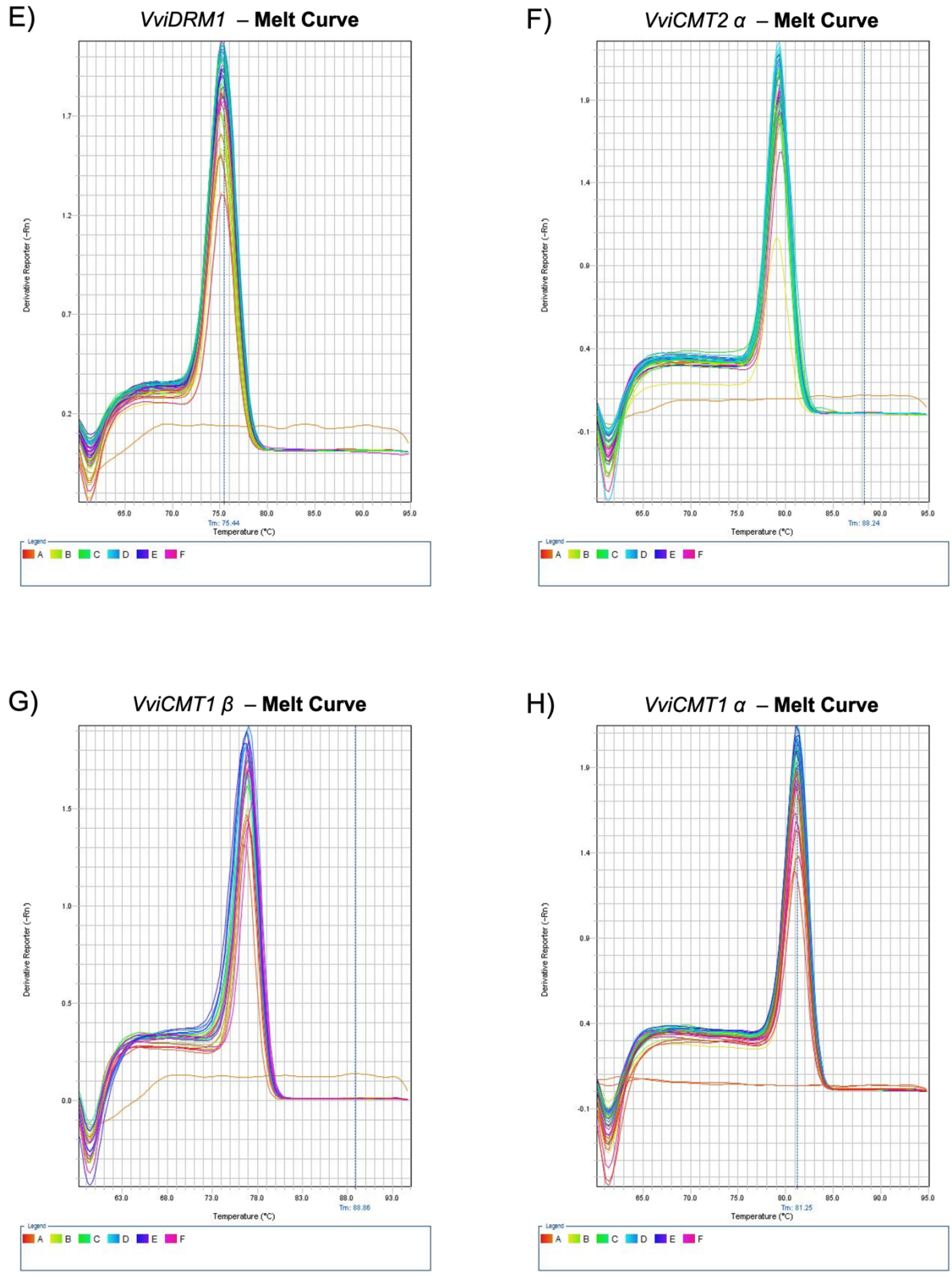
Adopted identifier	Exons	Introns	ORF Length (nt | aa)
VviDNMT1	10	9	1182 | 393
VviDRM1	11	10	1809 | 602
VviDRM2 δ	12	11	2205 | 734
VviDRM2 β	12	11	2151 | 716
VviDRM2 γ	12	11	2139 | 712
VviDRM2 ε	12	11	2097 | 698
VviDRM2 α	12	11	1770 | 589
VviCMT1 α	21	20	2490 | 829
VviCMT2 α	24	23	2679 | 892
VviCMT2 ε	25	24	4299 | 1432
VviCMT2 δ	25	24	4293 | 1430
VviCMT2 γ	25	24	4050 | 1349
VviCMT2 β	25	24	4095 | 1364
VviCMT1 β	22	21	2898 | 965
VviMET1	12	11	4650 | 1549
VviMET2 α	11	10	4650 | 1549
VviMET2 β	11	10	4710 | 1569

6.2. Appendix 2

Supplementary data 1 – Melting curves of reference and targeted genes: A) *SAND*; B) *EF1α*; C) *VviDNMT1*; D) *VviDRM2 δ*; E) *VviDRM1*; F) *VviCMT2 α*; G) *VviCMT1 β*; H) *VviCMT1 α*; I) *VviMET1*; J) *VviMET2 α*.

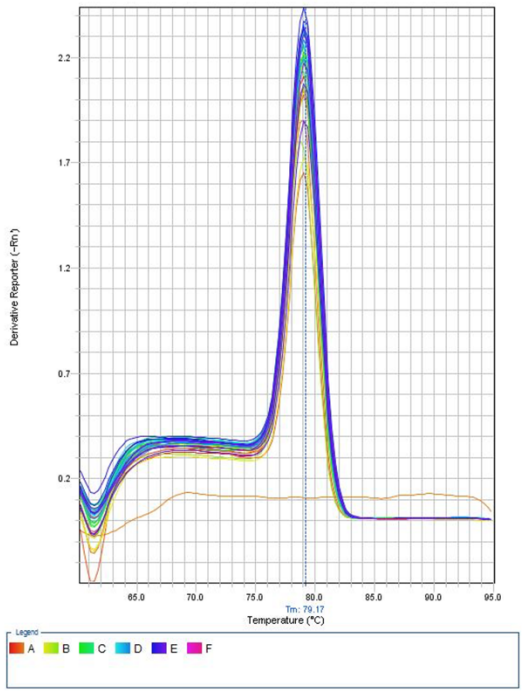


Supplementary data 1 (cont.) – Melting curves of reference and targeted genes: A) *SAND*; B) *EF1α*; C) *VviDNMT1*; D) *VviDRM2 δ*; E) *VviDRM1*; F) *VviCMT2 α*; G) *VviCMT1 β*; H) *VviCMT1 α*; I) *VviMET1*; J) *VviMET2 α*.



Supplementary data 1 (cont.) – Melting curves of reference and targeted genes: A) *SAND*; B) *EF1α*; C) *VviDNMT1*; D) *VviDRM2 δ*; E) *VviDRM1*; F) *VviCMT2 α*; G) *VviCMT1 β*; H) *VviCMT1 α*; I) *VviMET1*; J) *VviMET2 α*.

I) *VviMET1* – **Melt Curve**



J) *VviMET2 α* – **Melt Curve**