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Trabalho efetuado sob a orientação do
Doutor Artur Jorge da Silva Conde
e do
Professor Doutor Hernâni Varanda Gerós

dezembro de 2019

DECLARAÇÃO

DIREITOS DE AUTOR E CONDIÇÕES DE UTILIZAÇÃO DO TRABALHO POR TERCEIROS

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"At times, our own light goes out and is rekindled by a spark from another person. Each of us has cause to think with deep gratitude of those who have lighted the flame within us." – Albert Schweitzer

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STATEMENT OF INTEGRITY

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

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Abstract

The global quality and characteristics of grape berries, and ultimately of wine, are influenced by their polyphenolic composition. Therefore, potential strategies to improve berry quality by targeting secondary metabolism pathways of phenolic compound synthesis are useful, particularly in an ongoing context of climate change. These pathways are modulated by several molecular mechanisms, including regulation of gene transcription by specific transcription factors and post-transcriptional regulation by microRNAs. Recently, it was discovered that the primary (non-mature) miRNAs transcripts (pri-miRNAs) could encode for small regulatory peptides (micropeptides – miPEPs). In a positive loop, these miRNA-encoded peptides enhance the transcription and accumulation of their corresponding pri-miRNAs and, consequently, of their mature miRNAs, subsequently leading to an accentuated negative regulation of miRNA-regulated target genes.

The objective of this work was to explore this recent discovery and to experiment the exogenous application of a micropeptide (miPEP396a) that putatively promotes the inhibition of the transcription factor VvMYB5b, an activator of the expression of several genes involved in the flavonoid pathway. Designated in this study as miPEP-MYB5b, this micropeptide may serve as a fine-tuning tool for modulation of secondary metabolic pathways in grape berry cells and, consequently, improve their global quality-traits. MiPEP-MYB5b was identified *in silico* and exogenously added to a Gamay Freaux grape berry cells in two different concentrations (0.1 µM and 0.5 µM). Its effect in the concentration of secondary metabolites such as anthocyanins, total flavonoids, total phenolics and stilbenes as well as in the transcription of key genes involved in biosynthetic routes that produce secondary metabolites with bioactive properties and important for grape berry quality, particularly in flavonoid- and stilbene-synthesizing pathways was analyzed. Both concentrations of miPEP-MYB5b resulted in downregulation of key genes involved in the flavonoid pathway, such as *VvLAR1, VvLAR2,* and *VvCHI*, while 0.5 µM resulted in downregulation of flavonoid-related genes *VvANR, VvFLS1, VvCHS1.* A parallel stimulation of the expression of the stilbene-synthesizing gene *VvSTS1* was also observed in miPEP-treated cells. This upregulation of the stilbene pathway was probably due to a miPEP-MYB5b-mediated inhibition of MYB5b and, thus, of the flavonoid pathway, that competes directly with the stilbene pathway for substrate. Concordantly with the inhibition of the flavonoid pathway and stimulation of the stilbene pathway, a higher stilbene content and lower concentration of flavonoids (including anthocyanins) were quantified in grape berry cells. Thus, miPEP-MYB5b exogenous application may be a promising strategy to modulate secondary metabolic pathways in order to produce and accumulate higher quantity of stilbenes in grape berry cells in a near future, by exploring mechanisms of microRNA-mediated gene regulation in plants.

Resumo

A qualidade e características globais dos bagos de uva e do vinho são influenciadas pela composição em polifenólicos. Por isso, estratégias que melhorem a qualidade dos bagos tendo como alvo as vias do metabolismo secundário que sintetizem compostos fenólicos são úteis, particularmente, no contexto atual das alterações climáticas. Estas vias são moduladas por vários mecanismos moleculares, incluindo a regulação da transcrição de genes através de fatores de transcrição específicos e regulação pós-transcricional através de microRNAs. Recentemente, descobriu-se que os transcritos primários (não-maduros) dos miRNAs codificam pequenos péptidos reguladores (micropéptidos- miPEPs). Num "*loop*" positivo, estes micropéptidos aumentam a transcrição e a acumulação do pri-miRNA e miRNA correspondentes e, posteriormente, levam a uma regulação negativa dos genes alvo de uma forma mais acentuada.

O objetivo deste trabalho consistiu em explorar esta recente descoberta e experimentar a aplicação exógena de um micropéptido (miPEP396a) que, putativamente, promove a inibição do fator de transcrição VvMYB5b, um ativador da expressão de vários genes envolvidos na via dos flavonoides. Designado por miPEP-MYB5b neste estudo, este micropéptido poderá permitir a manipulação das vias do metabolismo secundário nas células dos bagos e, consequentemente, melhorar a sua qualidade global. O miPEP-MYB5b foi identificado *in silico* e depois adicionado a uma cultura celular de bagos da variedade *Gamay Freaux*, em duas concentrações diferentes (0,1 µM e 0,5 µM), sendo analisado o seu efeito na concentração de metabolitos secundários como antocianinas, flavonoides totais, fenólicos totais e stilbenos, bem como na transcrição de genes-chaves envolvidos nas vias metabólicas que produzem estes compostos secundários com propriedades bioativas e de grande importância para a qualidade dos bagos, particularmente nas vidas de síntese de flavonoides e stilbenos. Ambas as concentrações de micropéptido levaram à regulação negativa dos genes *VvLAR1, VvLAR2* e *VvCHI*, enquanto a 0,5 µM provocaram uma regulação negativa de genes relacionados com a via dos flavonoides, como *VvANR, VvFLS1* e *VvCHS1.* Foi também observada uma estimulação da expressão do gene *VvSTS1*, responsável pela síntese de stilbenos, em células tratadas com o micropéptido. Esta regulação positiva da via dos stilbenos ocorreu, provavelmente, devido a uma inibição do MYB5b mediada pelo micropéptido que, em consequência, inibiu a via dos flavonoides, que compete por substrato diretamente com a via dos stilbenos. Para além da inibição da via dos flavonoides e estimulação da via dos stilbenos, detetaram-se maiores quantidades de stilbenos e menor concentração de flavonoides (incluindo antocianinas) em células de bagos de uva. Desta forma, a aplicação exógena do miPEP-MYB5b poderá ser uma estratégia promissora para modular as vias do metabolismo secundário para produzir e acumular mais stilbenos em células dos bagos de uva, explorando os mecanismos de regulação genética mediados por miRNAs em plantas.

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List of abbreviations and acronyms

4CL	4-coumaroyl:coA-ligase
ABCC1	ATP binding cassette subfamily C member 1
ACT	Actin
AGO1	Argonaute protein 1
ANR	Anthocyanidin reductase
Beta-MCD	beta-methyl cyclodextrin
C4H	Cinnamate-4-hydroxylase
CCD4	Carotenoid cleavage dioxygensase
CHI	Chalcone isomerase
CHS	Chalcone synthase
DCL1	Dicer-like 1
DFR	Dihydroflavonol reductase
DW	Dry weight
F3'-5'H	Flavonoid-3'-5'-hydroxylase
F3'H	Flavonoid-3'-hydroxylase
F3H	Flavanone-3-hydroxylase
FLS	Flavonol synthase
GAE	Gallic acid equivalents
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GST4	Glutathione S-transferase 4
HEN1	S-adenosyl methionine-dependent methyltransferase
HST1	Plant orthologue of exportin-5, HASTY
LAR	Leucoanthocyanidin reductase
LDOX	Leucoanthocyanidin dioxygenase
MATE1	Multidrug and extrusion protein 1
MeJa	Methyl jasmonate
miPEP	miRNA-encoded peptide
miRNA	microRNA
NAA	α- Naphthaleneacetic acid
OMT	O-methyl transferase

ORF	Open reading frame
PA	Proanthocyanidin
PAL	Phenylalanine ammonia lyase
PCR	Polymerase chain reaction
pre-miRNA	percursor of miRNA
pri-miRNA	primary transcript of miRNA
qPCR	Quantitative real-time PCR
RISC	RNA-induced silencing complex
SEM	Standard error of mean
siRNA	Small interfering RNA
STS	Stilbene synthase
UFGT	UDP-glucose-flavonoid-3-*O*-glucosyltransferase

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1. INTRODUCTION

1. Introduction

1.1. Cultivation and economic importance of *Vitis vinifera* L.

Numerous *Vitis* species have great economic importance due to their role in production of wine, table grapes and other products. Among all the species in Vitaceae family, the most important one is *Vitis vinifera* L., commonly known as grapevine, because of its widespread use for wine production (Rivière et al., 2012). Grapevine is a perennial woody plant and is one of many important fruit crops. According to records, domesticated grape cultivation began 6,000-8,000 years ago and it has been a part of human history since then (McGovern, 2003; Wong et al., 2016). The majority of investigations that have been performed in grapevine were due to its human health and overall economic value (Rivière et al., 2012). Grape berries provide numerous secondary metabolites with well-known health benefits such as phenolic compounds, like flavonoids and stilbenes, with strong bioactive properties. In addition, the global quality and characteristics of grape berries and, ultimately wine, are affected by the overall polyphenols composition (Tavares et al., 2013). Indeed, several factors, such as environmental conditions and viticulture practices, affect the total polyphenolic composition and lead to molecular difference in grapes (Teixeira et al., 2013).

In recent years, there have been more records of periods of extreme elevated temperatures which ultimately affect grapevine cultivation. Therefore, climate changes in the future not just threaten grape berries development and quality, but also greatly impact the overall quality of wine. In this adverse context, besides the optimization of viticulture practices, the use of molecular modulation strategies could be a step to preserve or even increase grape berry synthesis of quality-related metabolites and overall quality and nutritional value.

1.2. Grape berry phenolic compounds

Even though plant secondary metabolites, such as phenolic compounds, have no crucial role in plant life maintenance processes, they are critical for plant interactions with its environment, for adaptation and defense mechanisms (Ramakrishna & Ravishankar, 2011; Zhao et al., 2013). Besides their biological functions, secondary metabolites have a big importance in human life because they are a particular source for food additives, flavors, pharmaceuticals, fragrance ingredients and pesticides (Ramakrishna & Ravishankar, 2011; Rao et.al, 2011; Vanisree et al., 2004).

Phenolic compounds are the most extensive group of secondary metabolites and consist of a phenyl ring backbone with hydroxyl groups or other substitutes. Grape phenolics give color, flavor,

astringency, texture and antioxidant properties to wine. This class of compounds are divided between nonflavonoid and flavonoid compounds. Nonflavonoids includes hydroxybenzoic acids, hydroxycinnamic acids, volatile phenols and stilbenes while flavonoids includes flavones, flavonols, flavanones, flavan-3-ols and anthocyanins (Teixeira et al., 2013).

1.2.1. Nonflavonoid compounds

Phenolic hydroxycinnamic acids are usually accumulated in the skin and in the flesh of red and white berries. After proanthocyanidins and anthocyanins, the hydroxycinnamates are the most abundant class of soluble phenolics in grape. The most predominant hydroxycinnamates are *p*-coumaric, caffeic and ferulic acids and its accumulation occurs in all berry tissues. Hydroxycinnamates may be conjugated with anthocyanins in hypodermal, mesocarp and placental cells of the pulp. Hydroxybenzoic acids are normally lowly present in wine when compared to hydroxycinnamates and the most common are salicylic acid, gallic acid, gentistic acid and p-hydroxybenzoic. Salicylic acid is involved in signaling in plants, especially in the induction of stress defense and responses (Teixeira et al., 2013).

Stilbenes are present mainly in the skin and seeds in grape berries and are also present in low quantities in wine. These compounds have been drawing the attention of scientific research due to their therapeutic properties, particularly resveratrol (Teixeira et al., 2013). *Trans*-resveratrol (3,5,4'-trihydroxytilbene) is the stilbene with the simplest molecular structure, which is used as precursor for other compounds through various modifications such glycosylation or methylation. In grapevine, the many stilbene compounds includes resveratrol glucosides like piceid (trans- and *cis*-resveratrol- 3-O-β-D-glucopyranoside), methoxylated stilbenes like pterostilbene (trans -3,5-dimethoxy-4'-hydroxy-stilbene) and oligomers like viniferins (Chong et. al, 2009; Roubelakis-Angelakis, 2009). Viniferins are the major group of resveratrol oligomers produced by oxidation of basic stilbenes. The most important viniferins are α- β- γ- δ- ε-viniferins, composed essentially by cyclic oligomers of resveratrol.

1.2.2. Flavonoid compounds

Flavonoids compose a substantial portion of the phenolic compounds in grapes and comprise several classes. They are C6-C3-C6 polyphenolic compounds where two hydroxylated benzene rings, A and B, are connected by a three-carbon chain that belongs to a heterocylic C ring (figure 1) (Conde et al., 2007).

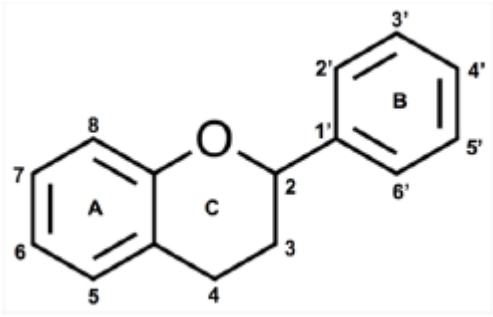

Figure 1. Flavonoid ring structure and numbering (Teixeira et al., 2013).

Grape flavonoids are confined mainly to both peripheral layers of berry skin and in some layers of the seed coat. There, the considerable amount of flavonoids is mainly composed of flavan-3-ols, flavonols, proanthocyanidins and anthocyanins.

Flavonols have a 3-hydroxyflavone backbone and they differ by the number and type of substituents on the B ring. This class of flavonoids occurs typically as glucosides, glucuronides, galactosides and rhamnosides with a sugar linked to the 3 position of the flavonoid skeleton. The grape berry produces kaempferol, myrcetin, quercetin and methylated forms isoharmnetin, laricitrin and syringetin. Quercetin plays a role in co-pigmentation with anthocyanins and are also known to act as UV-protectants. In addition, total flavonols concentration in berry can be powerfully affected by environmental factors, specially sunlight exposure (Teixeira et al., 2013).

In grape berry, the most abundant class of phenolic compounds are flavan-3-ols. They have monomeric structures like catechins and polymeric structures known as proanthocyanidins (PAs, also known as condensed tannins). Catechins and PAs are primarily present in seeds, then in berry skin and very little in the pulp. Catechins are identified by the presence of a hydroxyl group at the 3 position of the C ring and they are associated with bitterness and astringency of wine (Teixeira et al., 2013). Proanthocyanidins are the most abundant class of soluble polyphenols in grape berries. These compounds are responsible for organoleptic properties including astringency, bitterness and long-term color stability of grape berries and wine. PAs differ in size, reaching from dimers to polymers with more than 40 units, depending where they are located. For example, grape proanthocyanidins have greater average size on the skin than in seeds. Grape proanthocyanidins contain the flavan-3-ol subunits (+)-catechin (C), (-)-epicatechin (EC), (-) epicatechin-3-*O*-gallate (ECG), and (-)-epigallocatechin (EGC) (Conde et al.,2016; Cortell et al., 2005).

Anthocyanins cause red, purple and blue pigmentation in grape berries and, consequently, in red wine. The core of this compounds is the flavylium, a typical C6-C3 skeleton. Basically, anthocyanins are

glycosides and acylglycosides of anthocyanidins. In grapevine, there are six anthocyanidins reported: pelargonidin, cyanidin, peonidin, delphinidin, petunidin and malvidin. Anthocyanins can also be esterified by acids like acetic, coumaric or caffeic, connecting the 6' position of the glucose to the 3' position of the C ring. In grapevine, the anthocyanins normally found are delphinidin, cyanidin, petunidin, peonidin and malvidin 3-glucosides, 3-(6-acetyl)-glucosides and 3-(6-p-coumaroyl)-glucosides and peonidin and malvidin 3-(6-caffeoyl)-glucosides. Malvidin-3-O-glucoside and its acylated forms are usually the major anthocyanins present in grape berry. In addition, anthocyanins are produced in cytosol of the epidermal cells and then stored in the vacuole. They are also co-localized with proanthocyanidins in skin hypodermal layers (Conde et al., 2007; Teixeira et al., 2013).

1.3. Bioactive and therapeutic properties of phenolic compounds

As mentioned before, many grape berry secondary metabolites possess bioactive functions and beneficial effects on humans. Anthocyanins, proanthocyanidins and stilbenes are among the compounds with significant bioactive and therapeutic properties. Grape anthocyanins are induced by biotic and abiotic stress factors, including radiation, high or low temperatures and microbial and viral attacks. The major roles of anthocyanins in grape berries are protection against solar exposure and UV radiation and free radical scavenging, antioxidant, bactericidal, antiviral, and fungistatic activities. Similarly, in humans, they have radical scavenging and antioxidant properties, antimicrobial, antiviral, anticancer and antimutagenic activities and provide protection against cardiovascular disease and hepatic damage (Ananga et al., 2013; Delgado-Vargas et al., 2010; He et al., 2010). Proanthocyanidins' major function is to provide plant protection against microbial pathogens, insect pests and herbivory, as reviewed by Dixon et al. (2005). PAs have multiple pharmacological benefits, including antibacterial, antiviral, antimutagenic, antitumoral, antioxidant and anti-inflammatory activities, cardiovascular risk-reducing properties and attenuation of arteriosclerosis (Iriti et al., 2005; Yamakoshi et al., 1999).

Stilbenes play key roles in plant protection and adaptation against environmental stresses. This class of compounds exhibit biological roles in plants in response to wounding or pathogen attack, showing antifungal activity, antimicrobial properties, nematocidal activity and insecticidal activity (Jeandet et al., 2016; Lieutier et al., 1996; Suga et al., 1993; Torres et al., 2003). The stilbene resveratrol is linked to the "French paradox" and was first isolated from the white hellebore (Takaoka, 1940). This compound is present in red wine and its daily consumption has been connected to several health benefits such as antioxidant, cytoprotective, antiaging and anticancer properties, neuroprotective

effect against Alzheimer's disease and prevention of cardiovascular diseases and diabetes. Furthermore, this compound displayed its capacity to extend lifespan in metazoans and mice (Jeandet et al., 2016; Waffo-Teguo et al., 2017). In addition, Baur and Sinclair (2006) explained resveratrol effects that have been observed *in vivo* and that could possibly be involved in development of human therapeutics. It is also widely used in the cosmetics industry as an active ingredient of anti-aging beauty creams.

1.4. Metabolic pathways involved in the synthesis of phenolics

All flavonoid and nonflavonoid compounds are synthesized via the universal phenylpropanoid pathway (**figure 2**). It begins with phenylalanine as substrate, that is converted to trans-cinnamic acid by phenylalanine ammonia lyase (PAL), that is subsequently converted by cinnamate-4-hydroxylase (C4H) in *para*-coumaric acid. The 4-coumaroyl:CoA ligase (C4L) catalyzes the ligation of *para*-coumaric acid to a coenzyme A to produce *para*-coumaroyl-CoA. Following this, chalcone synthase (CHS) and stilbene synthase (STS) compete for this substrate to condense it with three units of malonyl-CoA directing the production of naringenin chalcone, the first intermediate in the flavonoid pathway, in the first case; and resveratrol, the first intermediate in the stilbenoid pathway, in the second case (Roubelakis-Angelakis, 2009). All flavonoids derive from tetrahydroxychalcone and, as previously mentioned, the flavonoid pathway leads to the production of different flavonoids, including proanthocyanidins, flavonols, anthocyanins and flavan-3-ols. On the other pathway, the first step of the stilbene pathway is regulated by STS. The competition of STS and CHS for the same substrate commands the entry point into the flavonoid or stilbene pathways (Teixeira et al., 2013).

1.5. Molecular regulation of flavonoid pathway

The transcriptional regulation of flavonoid biosynthetic pathway is mainly controlled by MYB and basic helix-loop-helix (bHLH) together with WD40 proteins. Transcription factors of the bHLH type can modulate, sometimes in an overlapping way, one or more branches of the flavonoid pathway. However, most of the MYB transcription factors characterized to date regulate only one branch of the flavonoid pathway (Hichri et al., 2011).

In grapevine, many transcription factors are involved in the regulation of the flavonoid pathway by inducing or silencing key biosynthetic genes along the flavonoid pathway. For example, VvMYBA1 transcription factor controls the expression of *VvUFGT1*, inducing anthocyanins synthesis during ripening of the berries (Cutanda-Perez et al., 2009); overexpression of *VvMYB5a* in tobacco (*Nicotiana*

tabacum) increased the biosynthesis of condensed tannins (Deluc et al., 2006); overexpression of *VvMYB5b* in tobacco lead to accumulation of anthocyanin- and proanthocyanidin-derived compounds (Deluc et al., 2008); VvMYBPA1 activates the promoters of *VvLAR* genes and *VvANR*, as well as the promoters of several general flavonoid pathway genes leading to proanthocyanidin accumulation (Bogs et al., 2007) and ectopic expression of either *VvMYBPA1* and *VvMYBPA2* activated enzymes of the flavonoid pathway, including the specific terminal steps in the biosynthesis of epicatechin and catechin by activating anthocyanidin reductase and leucoanthocyanidin reductase 1 (Terrier et al., 2009).

1.6. Molecular regulation of stilbene pathway

Stilbenes contemplate the most important phytoalexin group in grapevine. Phytoalexins production and accumulation in plants occurs in response to biotic and abiotic stresses. In grapevine, stilbene biosynthesis can be induced by biotic stress factors that include downy mildew (*Plasmopara viticola*), gray mould (*Botrytis cinerea*), and some *Aspergilli* spp. So, the stress caused by fungal attack induces production of resveratrol and its derivates as a grapevine defense mechanism (Roubelakis-Angelakis, 2009). Hormonal signaling and abiotic stress factors like UV- radiation, aluminium, ozone, methyl jasmonate (MeJa), benzothiadiazole, salicylic acid, mineral nitrogen depletion, water deficit stress and sulfate depletion also induce stilbene synthesis (Roubelakis-Angelakis, 2009;Tavares et al., 2013). Therefore, artificial biosynthesis of this compounds can be induced by treating cells with elicitors that imitate a pathogen attack in order to induce their defense mechanisms (Ascensión Martínez-Márquez et. al, 2016, Adrian and Jeandet, 2012, Donnez et al., 2011). Lijavetzky and his group revealed that the effect of MeJa on cell division together with a strong elicitor such as cyclodextrin powerfully induces resveratrol production (Lijavetzky et al., 2008) and, more recently, jasmonic acid, salicylic acid, β-glucan and chitosan displayed ability to enhance intracellular resveratrol biosynthesis and the combination of jasmonic acid with β-glucan induces extracellular resveratrol production (Vuong et al., 2014).

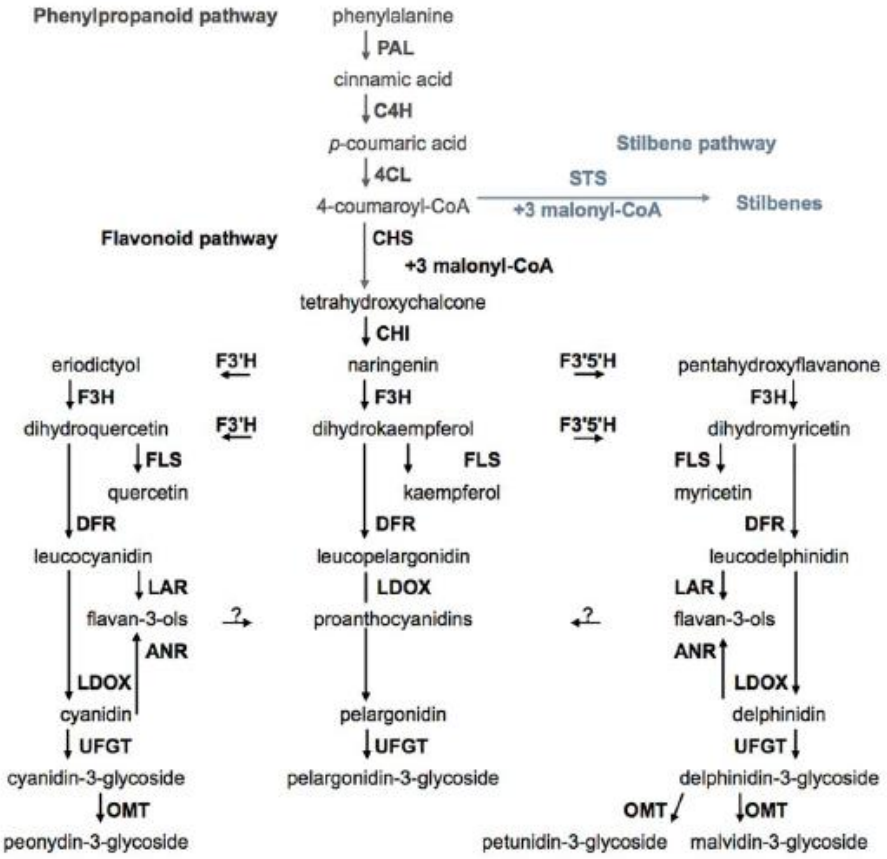

Figure 2. Phenolic metabolism pathways in grape berry. Phenylalanine ammonia lyase (PAL), cinnamate-4-hydroxylase (C4H), 4-coumaroyl:CoA-ligase (4CL), stilbene synthase (STS), chalcone synthase (CHS), chalcone isomerase (CHI), flavonoid 3'-hydroxylase (F3'H), flavonoid 3',5'-hydroxylase (F3'5'H), flavanone-3-hydroxylase (F3H), flavonol synthase (FLS), dihydroflavonol reductase (DFR), leucoanthocyanidin reductase (LAR), anthocyanidin reductase (ANR), leucoanthocyanidin dioxygenase (LDOX), dihydroflavonol 4-reductase (DFR), flavonoid glucosyltransferase (UFGT), O-methyltransferase (OMT) (He et al., 2010; Teixeira et al., 2013).

The grapevine genome sequencing offered numerous lines of evidence for growing gene families related to wine quality and health benefits. Between these, information has been unveiled of genes involved in the synthesis of secondary metabolites, including stilbene synthase family (Wong et al., 2016). Recently, it was reported that a considerable number of transcription factors linked to different families such WRKYs, MYBs and ERFs might be present in STS regulation. In fact, *VvMYB13*, *VvMYB14*, *VvMYB15*, *VvWRKY3*, *VvWRKY24*, *VvWRKY43* and *VvWRKY53* are co-expressed with *VvSTS* genes in biotic and abiotic stresses (Vannozzi et al., 2018; Wong et al., 2016). A negative system modulator of resveratrol biosynthesis composed by VvMYB14, VvWRKY8, VvSTS15/21 and resveratrol itself has also been revealed. Basically, VvWRKY8 interacts with the N-terminus of VvMYB14 which prevents VvMYB14 interaction with *VvSTS15/21* promoter. In addition, resveratrol exogenous application in cell suspension cultures considerably enhanced *VvWRKY8* expression and reduced *VvMYB14* expression (Jiang et al., 2018). Possibly, several other transcription factors can regulate this pathway indirectly, since it's a very

complex route where many transcriptions factors and other regulatory proteins interact to regulate several pathways.

1.7. Manipulation of stilbene production using micro-RNA encoded micropeptides – a viable strategy?

In plants, microRNAs (miRNAs) regulate the expression of several different genes and, consequently, a numerous of biological functions, such as hormonal control, adaptation to various biotic and abiotic stresses and immune responses (Flynt and Lai, 2008). MicroRNAs are short sequences that are synthesized by enzymatic excision from precursor transcripts known as primary pri-miRNAs (Waterhouse & Hellens, 2012). A recent investigation revealed that these pri-miRNAs have the capacity to encode small regulatory peptides, also called micropeptides (miPEPs), representing a very promising strategy for gene regulation without the need for genetic transformation (Lauressergues et al., 2015).

1.7.1. Micro-RNAs biogenesis

Micro-RNAs are a class of small and non-translated RNAs that have a regulatory role in post-transcriptional gene silencing. MicroRNAs are chemically and functionally similar to small interfering RNAs (siRNAs). Both are processed by Dicer-like RNase III family of enzymes but instead of being processed from long and double-stranded precursors like siRNAs, miRNAs derive from local stem-loop structures. Both miRNAs and siRNAs are incorporated into silencing complexes that comprise Argonaute proteins where they can promote repression of target genes. In addition, plant micro-RNAs are extremely complementary to conserved target mRNAs. Thus, it's possible to run a fast and assured bioinformatic identification of plant miRNAs targets (Jones-Rhoades et al., 2006).

In plants, miRNAs are 20-24 nucleotide RNA molecules that regulate gene expression post-transcriptionally. MicroRNAs use base pairing to guide RISCs (RNA-induced silencing complexes) to specific messages carrying fully or partly complementary sequences. Most of MIR genes are RNA polymerase II (pol II) transcription units that produce the primary miRNA transcript called pri-miRNA (**figure 3**). The pri-miRNA naturally forms an imperfect fold-back structure, which is processed into a stem-loop precursor (pre-miRNA). After this, the pre-miRNA is excised as an RNA duplex. The pri-to-pre-miRNA conversion and miRNA maturation are performed by Dicer-like 1 (DCL1). Mature miRNA duplexes are stabilized by the S-adenosyl methionine- dependent methyltransferase Hua Enhancer 1 (HEN1) by methylation of plant-silencing small RNAs. The plant orthologue of exportin-5, HASTY (HST1), is also required for miRNA function and biogenesis (Voinnet, 2009).

1.7.2. Micro-RNAs mode of action in gene regulation

The miRNA-mediated gene regulation can occur in two theoretically distinguishable modes (**figure 3**). In the first (**figure 3**, left), micro-RNAs can repress target protein translation in a reversible manner. In theory, this type of modulation can coordinate and reset stress-responsive gene expression. With this, the cell can safeguard that translation of negative regulators resumes immediately after the stress, so the length of stress response isn't more than required. On the other hand, in the second mode (**figure 3**, right) miRNAs operate through transcript cleavage. This step requires 100% complementary between the miRNA and the target and produces irreversible switches that are necessary to establish permanent cell fates (Voinnet, 2009).

1.7.3. Micropeptides - Discovery and its importance in gene regulation

The miRNAs primary transcripts were first considered to be "junk RNA", unable to encode for any proteins. However, Lauressergues and colleagues (Lauressergues et al., 2015) revealed evidence of the contrary, showing its ability to encode small regulatory peptides. In the intergenic regions of a genome are the sequences that lead to the production of most pri-mRNAs. These authors recognized short open reading frames (ORFs) in many different pri-miRNAs and revealed that the ORFs are naturally translated into peptides, called micropeptides (miPEPs). Even if the mechanisms underlying if pri-miRNAs undergo either processing into mature miRNA or into miPEPs are still not fully understood, this discovery exposes an unexpected function of pri-miRNAs sequences and reveals another layer of gene regulation in plant cells (Lauressergues et al., 2015; Waterhouse and Hellens, 2012).

The synthesized microRNA-encoded peptides trigger the transcription of their associated mature miRNAs, leading to the synthesis of higher quantity of miRNA and a more noticeable silencing of corresponding target genes. Remarkably, plant treatments with synthetic miPEPs can strongly affect plant phenotype due to this positive regulation of corresponding mature miRNAs. This fact brings to light a possible strategy of gene modulation via exogenous treatments of easy implementation on agronomical practices. An array of *in silico* searches and molecular analyses enable the identification of miPEPs that putatively regulate target genes of interest so they can be downregulated by exogenous application of such miPEPs, thus, without the need for genetic transformation of crops (Couzigou et al., 2015).

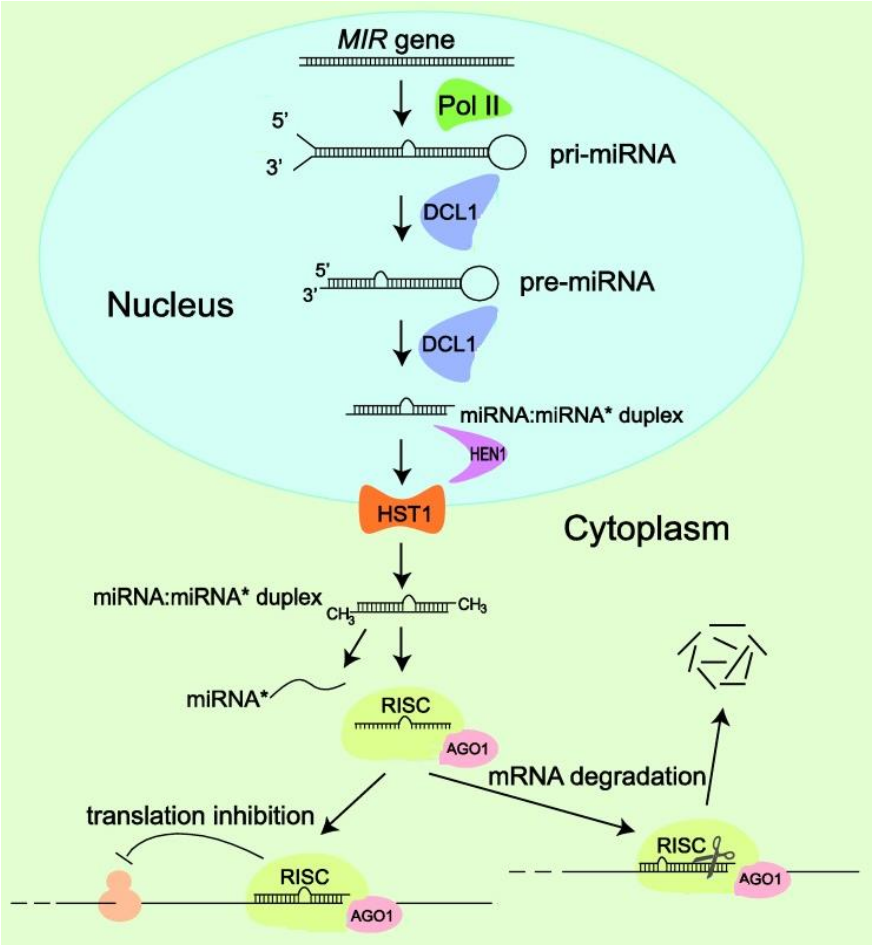

Figure 3. MicroRNA biogenesis in plants. MIR genes are firstly transcribed by Pol II into pri-miRNAs and are folded to form hairpin structure. Pri-miRNAs and pre-miRNAs are consecutively processed by DCL1 (Dicer-like 1) to produce one or many phased miRNA/miRNA* duplexes, which are methylated by HEN1 (S-adenosyl methionine- dependent methyltransferase Hua Enhancer 1) and transported to cytoplasm by HST1 (plant orthologue of exportin-5, HASTY). The miRNA is selected and incorporated into AGO1 (Argonaute proteins) containing RISC (RNA-induced silencing complexes) that directs either translation inhibition or cleavage of the target mRNA transcript. Adopted from Yang and Li, (2014).

1.8. Objectives

The recent study of Lauressergues and colleagues (Lauressergues et al., 2015) also revealed that the overexpression of miPEP171b in *Medicago truncatula* stimulated the accumulation of endogenous miR171b, resulting in differences in root development. Furthermore, in soybean (*Glycine max*), exogenous application of specific synthetic miPEPs demonstrated positive impact in nodule formation (Couzigou et al., 2016). Based on these data, miPEPs might also be used to modulate quality traits and concentration of certain compounds, thus potentially increasing crops agronomical value. In this sense, key genes involved in secondary metabolic pathways as well as transcription factors that tightly regulate such pathways might be promising targets of miPEP-mediated regulation towards the modulation of those pathways and increase/decrease of their metabolites synthesis, resorting to the exogenous addition of putative miPEPs as a tool.

As previously mentioned, Deluc and colleagues (Deluc et al., 2008) demonstrated that the grapevine transcription factor MYB5b activates the promoters of many structural genes of the flavonoid pathway when overexpressed in *Nicotiana tabacum*, leading to their up-regulation and greater anthocyanin and proanthocyanidin accumulation. Among others, this transcription factor up-regulates genes such as *VvLAR1*, *VvANR* and *VvANS*. Also, since the flavonoid (via CHS) and stilbene (via STS) pathways compete for the same substrate (4-coumaroyl-CoA), an inhibition of the flavonoid pathway might lead to the stimulation of the stilbene pathway as more substrate is available to enter this metabolic route via STS action. We identified *in silico* Vvi-miR396a as a miRNA putatively capable of negatively regulating *VvMYB5b*, that also had a possible corresponding miPEP (miPEP396a) encoded in its non-mature version. Thus, the micropeptide miPEP396a (also named in this study as miPEP-MYB5b, for facilitation purposes) might theoretically be a tool to increase grape berry cell concentration of stilbenes without genetic transformation, via miRNA-mediated inhibition of key molecular mechanisms involved in several branches of the flavonoid pathway. This can have significant impact as stilbenes are, as previously mentioned, important bioactive compounds with nutraceutical and antifungal properties with an important industrial application. In addition, the increased synthesis of stilbenes could hypothetically result in higher grapevine resistance against pathogen attacks.

Taking this into account, the main objective of this work was to confirm if the exogenous application of miPEP-MYB5b in grape berry cells of the variety Gamay leads to miRNA-mediated inhibition of the transcription factor VvMYB5b and consequent increase in the synthesis/accumulation of stilbenes as resveratrol, trans-piceid and viniferins, at the expense of a downregulation of flavonoid biosynthetic genes and lower intracellular flavonoid content. After validation of miPEP-MYB5b regulatory function, the experimentation of elicitation strategies towards increasing more significantly the *in vitro* production of stilbenes, using a strategy that combines the exogenous application of miPEP-MYB5b with methyl jasmonate (MeJA) and beta-methylcyclodextrin (beta-MCD) treatments in Gamay grape berry cells. This work has the potential for a scale-up for industrial/commercial purposes, as well as for the implementation of exogenous application in whole grape berries as a viticultural management practice towards for agronomical valorization.

2. MATERIAL AND METHODS

2. Material and Methods

2.1. *In silico* analysis for identification of the putative micropeptide miPEP396a (miPEP-*MYB5b*)

The work began with an array of *in silico* analyses to identify potential miPEPs in grapevine by combining several bioinformatic tools and databases. The bioinformatic tool psRNATarget Finder (Dai et al., 2018), a plant small regulatory RNA target predictor, with the aid of GenBank, was used to retrieve information on which, how and where (in the target RNA) miRNAs putatively regulated a key gene involved in the regulation of the flavonoid biosynthetic pathway: the transcription factor *VvMYB5b*. The identified mature miRNA of that target of interest was then screened in miRbase (microRNA database) (Kozomara and Griffiths-Jones, 2014) for its stem-loop sequence, the non-mature sequence of the regulatory miRNA possibly harboring small open reading frames (ORFs) corresponding to a putative regulatory miPEP. Finally, the obtained stem-loop sequences were then ran in a bioinformatic ORF finder tool that recognizes all possible ORFs that could translate into a small peptide, by defining several parameters based on the few miPEPs so far identified in the literature (Lauressergues et al., 2015), such as the need to have a start and a stop codon and a maximum length of encoding peptides of 20 amino acids. The result of these *in silico* analyses is resumed in **table 2**, shown in the Results section.

2.2. Solubilization of miPEP-*MYB5b*

Following their *in silico* identification, micropeptides were ordered from Smart Bioscience as 1 mg aliquots. Solubilization of the peptides was conducted as recommended by Smart-Bioscience Peptide Solubility Guidelines (https://www.smart bioscience.com/support/solubility/). The miPEP-MYB5b putatively negatively regulating *VvMYB5b* was solubilized in 600 µL of acetic acid (10%) and 400 µL of DMSO, so the final concentration of miPEP was 1 mg/mL. A solution of 600 µL of acetic acid (10%) and 400 µL of DMSO (equivalent to that used for miPEP-MYB5b), but without the peptide)) was used as control in the exogenous treatments.

2.3. Biological material

Grape berry cell suspensions of the cultivar Gamay Freaux were cultured in suspension in 30 mL of Gamborg B5 medium in 250 ml flasks at 25°C with constant agitation on a rotator shaker at 100 rpm and under 16h/8 h photoperiod. The culture medium composition was as follows: 3 g/L Gamborg

B5 salt mixture and vitamins; 30 g/L sucrose (3% m/v); 250 mg/L casein enzymatic hydrolysate; 0.1 mg/L α-napthaleneacetic acid (NAA) (200 mg/L); 0.2 mg/L kinetin (200 mg/L), and a final pH of 5.8. The suspension-cultured cells were cultivated for 10 d and then were subcultured by transferring 10 mL of cell suspensions to 30 mL of fresh medium.

2.4. Exogenous application of miPEP-MYB5b to Gamay grape cells

For each assay, immediately after sub-cultivation, 0.1 µM or 0.5 µM of miPEP-MYB5b were exogenously added to the cell cultures, in a volume that represented no more than 0.075 % (v/v) of the total volume of the cell suspension. All cell suspensions, including control cells (treated with the same volume of control solution) were cultivated for 10 d with constant agitation and a 16 h/8 h photoperiod. Cells were then collected, filtered and immediately frozen with liquid nitrogen and stored at -80 °C. A part of cells of each experimental condition was lyophilized. Cells were also harvested immediately before treatment which corresponds to the experimental condition t=0. Culture media of each experimental condition was also obtained via filtration and adequately stored at -20 °C.

2.5. Quantification of total phenolics

The concentration of total phenolics was performed as described in (Conde et al., 2016). The concentration of total phenolics was quantified by the Folin-Ciocalteu colorimetric method in grape cells from all experimental conditions (Singleton and Rossi, 1965). Total phenolics were extracted in 1 mL of a solution of 90% methanol and 10% water from about 100 mg of berry ground tissue. The homogenates were vigorously shaken for 30 min and subsequently centrifuged at 18000 *x g* for 20 min. Forty μL of each supernatant were added to 1.56 mL of deionized water and 100 μL of Folin reagent, vigorously shaken and incubated for 5 min in the dark before adding 300 μL of 2 M sodium carbonate. After 2 h of incubation in the dark, the absorbance of the samples was measured at 765 nm. Total phenolic concentrations were determined using a gallic acid calibration curve and represented as gallic acid equivalents (GAE). The concentration of total phenolics in medium cultures was performed with 100 μL of medium cultures added to 1.5 mL of deionized water, 100 μL of Folin reagent and 300 μL of 2 M sodium carbonate.

2.6. Quantification of anthocyanins

The concentration of anthocyanins was performed as described in (Conde et al., 2016). Anthocyanins were extracted from 100 mg of grape berry ground tissue with 1 mL of a solution of 90% methanol and 10% water. The suspension was vigorously shaken for 30 min. The homogenates were centrifuged for 20 min at 18000 *x g* and the supernatants were collected. Anthocyanin extracts were diluted 1:10 in 25 mM potassium chloride solution at pH 1.0 and the absorbance was measured at 520 nm and 700 nm, using 25 mM potassium chloride solution pH 1.0 as blank. Total anthocyanin quantification was calculated in relation to cyanidin-3-glucoside equivalent, calculated using the equation 1, per DW:

$$[Total\ anthocyanins](mg/L) = \frac{(A_{520}-A_{700})\times MW\times DF\times 1000}{\varepsilon\times 1} \quad (1)$$

where MW is the molecular weight of cyanidin-3-glucoside (449.2 g mol^{-1}), DF is the dilution factor and ε is the molar extinction coefficient of cyanidin-3-glucoside (26900 M^{-1} cm^{-1}).

2.7. Quantification of flavonoids

Quantification of flavonoids was performed according to the aluminum chloride colorimetric method described by Woisky and Salatino (1998), with some alterations. Quercetin was used to make the calibration curve. One milligram of quercetin was dissolved in 1 mL of 80% ethanol and then diluted to 5, 10, 25, 50, 75 and 100 μg mL^{-1}. For the reaction, the standard solutions (20 μL) and the methanolic grape berry cell extracts were separately mixed with 60 μL of 95% ethanol, 4 μL of 10% aluminum chloride, 4 μL of 1 M potassium acetate and 112 μL of distilled water. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 415 nm with a Shimadzu UV-160A spectrophotometer (Kyoto, Japan). The amount of 10% aluminum chloride was substituted by the same amount of distilled water in blank.

2.8. HPLC-DAD analysis

For high-performance liquid chromatography with diode-array detector (HPLC-DAD) analysis, methanolic extracts were filtered with 0.2 μm PTFE (Polytetrafluoroethylene) and then ran in a phenolic compounds specific column LichroCART column (Lichrospher 100. Rp-18e. 5μm, Merck, Germany)) with 1200 psi of pressure and flux of mobile phase of 0.8 mL/min. The compounds were eluted with two eluents, 0.1% formic acid in water and 0.1% formic acid in methanol, with diverse proportions throughout the method to produce a polarity gradient. The phenolic compound chromatograms at 284

nm, 304 nm and 520 nm were analyzed, as well as individual peaks/areas at various individual metabolite-specific retention times and maximum absorbance wavelengths.

2.9. Exogenous application of MeJa, beta-MCD and miPEP-MYB5b to Gamay grape cells for elicitation of phenolic compound synthesis

The combined effect of the exogenous application of miPEP-MYB5b in grape berry suspension-cultured cells together with an elicitation by methyljasmonate (MeJa) and beta-methylcyclodextrin was assessed by conducting an assay with Gamay grape berry suspension-cultured cells with the addition of 100 µM MeJa and 8.5 mM of beta-methylciclodextrin (double treatment) and another with 100 µM MeJa, 8.5 mM of beta-methylciclodextrin and 0.1 µM miPEP-MYB5b (triple treatment) in order to assess the potential of combining these possible stilbene-enhancing strategies.

2.10. RNA extraction and cDNA synthesis

Total RNA extraction was performed according to GeneJET Plant RNA purification Mini Kit (Thermo Scientific). After treatment with DNase I, cDNA was synthesized from 1 µg of total RNA Xpert cDNA synthesis Mastermix kit (Grisp), following the manufacturer's instructions. RNA concentration and purity were determined using Nanodrop.

2.11. Transcriptional analysis by real-time qPCR

Quantitative real-time PCR was performed with Xpert Fast SYBR (uni) Blue (Grisp) in a CFX96 Real-Time Detection System (Bio-Rad), using 1 µL of 1:10 diluted cDNA in a final reaction volume of 10 µL per well. Specific primer pairs used for each target gene are listed in **table 1**. Primers designed for amplification of non-mature vvi-miR396a were designed using the software Primer3Plus (Untergasser et al., 2007). Melting curve analysis was performed for specific gene amplification confirmation. As reference genes, *VvACT1* (actin) and *VvGAPDH* (glyceraldehyde-3-phosphate dehydrogenase) were selected, as these genes were proven to be very stable and ideal for qPCR normalization purposes in grapevine (Reid et al., 2006). For all experimental conditions tested, two independent runs with triplicates were performed. The expression values were normalized by the average of the expression of the reference genes as described by (Pfaffl, 2001) and analyzed using the software Bio-Rad CFX Manager (Bio-Rad).

2.12. Statistical analysis

The results were statistically analyzed by Student's t-test using Prism vs. 8 (GraphPad Software, Inc.). Statistical differences between mean values of each of the miPEP-MYB5b treatments and the Control are marked with asterisks.

Table 1. Primers forward (F) and reverse (R) used in qPCR studies for gene expression.

Gene	Accession number (Genoscope)	*Primers*
VvDFR1	GSVIVT01009743001	F: 5′-GGCTTTCTAGCGAGAGCGTA-3′
		R: 5′-ACTCTCATTTCCGGCACATT-3′
VvPAL1	GSVIVG01025703001	F: 5′-CCGAACCGAATCAAGGACTG-3′
		R: 5′-GTTCCAGCCACTGAGACAAT-3′
VvUFGT1	GSVIVT01024419001	F: 5'-TGCAGGGCCTAACTCACTCT-3'
		R: 5'-GCAGTCGCCTTAGGTAGCAC-3'
VvACT1	GSVIVT01026580001	F: 5'-GTGCCTGCCATGTATGTTGCC-3'
		R: 5'-GCAAGGTCAAGACGAAGGATA-3'
VvMATE	GSVIVT01028885001	F: 5'-TGCTTTTGTGATTTTGTTAGAGG-3'
		R: 5'-CCCTTCCCCGATTGAGAGTA-3'
VvCHS1	GSVIVT01032968001	F: 5′-GTCCCAGGGTTGATTTCCAA-3′
		R: 5′-TCTCTTCCTTCAGACCCAGTT-3′
VvGST4	GSVIVG01035256001	F:5'AAGGATCCATGGTGATGAAGGTGTATGGC-3'
		R:5'-AACTGCAGAAGCCAACCAACCAACAAAC-3'
VvCHS3	Lac14-SSH (CX126991)	F: 5'-TCGCATCACAAATAGCGAAC-3'
		R: 5'-CAGGGAAGCTGCCATGTATT-3'
VvABCC1	GSVIVT01028722001	F: 5'-CTCCACTGGTCCTCTGCTTC-3'
		R: 5'-AGCCTGCTTCGAAAGTACCA-3'
VvMYBPA1	GSVIVT01010590001	F: 5'-AGATCAACTGGTTATGCTTGCT-3
		R: 5'-AACACAAATGTACATCGCACAC-3
VvMYB5b	GSVIVT01025452001	F: 5'–CTCCATCCTTCATCTTCTTCA–3'
		R: 5'–GCCTTCTCCTTCTTTCTTGA–3'
VvANR	GSVIVG01035256001	F: 5'-CAATACCAGTGTTCCTGAGC – 3'
		R: 5'-AAACTGAACCCCTCTTTCAC – 3'
VvFLS1	GSVIVT01008913001	F: 5'-CAGGGCTTGCAGGTTTTTAG-3'
		R: 5'-GGGTCTTCTCCTTGTTCACG-3'
VvLAR1	GSVIVT01024419001	F: 5'-CAGGAGGCTATGGAGAAGATAC – 3'
		R: 5'-ACGCTTCTCTCTGTACATGTTG – 3'
VvLDOX	GSVIVT01009743001	F: 5'-ACCTTCATCCTCCACAACAT – 3'
		R: 5'-AGTAGAGCCTCCTGGGTCTT – 3'
VvGAPDH	GSVIVT00009717001	F: 5'-CACGGTCAGTGGAAGCATCAT-3'
		R: 5'-CCTTGTCAGTGAACACACCAG-3'
VvSTS1	DO366301	F: 5'- CGAAGCAACTAGGCATGTGT-3'
		R: 5′-CTCCCCAATCCAATCCTTCA-3′
VvCCD4b	GSVIVG01024554001	F: 5'-AAAGGGTGGGCAGTTCAGTT-3'

		R: 5'-GGGGGGTGAAAGGAAGATAT-3'
VvMYBA1	AB097923	F: 5′-GAGGGTGATTTTCCATTTGAT-3′
		R: 5′-CAAGAACAACTTTTGAACTTAAACAT-3′
VvLAR2	TC32909	F: 5'-TCTCGACATAAATGATGATGTG-3'
		R: 5'-TGCAGTTTCTTTGATTGAGTTC-3'
VvPRX31	VIT_14s0066g01850	F: 5'-ATGGCATTGATCCTCTTTTC – 3'
		R: 5'- CTAGTTTAAGGCATCACACC-3'
VvC4H	GSVIVG01024554001	F: 5'-AAAGGGTGGGCAGTTCAGTT-3'
		R: 5'-GGGGGGTGAAAGGAAGATAT-3'
VvCHI	X75963	F: 5'-GCGGATTCGGTTGACTTTTT-3'
		R: 5'-CTGGTAGGGACCCATCTTTG-3'
VvF3'5'H	AJ880356	F: 5'-AAACCGCTCAGACCAAAACC-3'
		R: 5'-ACTAAGCCACAGGAAACTAA-3'
miR396a	MI0006569	F: 5'- TGTCATGCTTTTCCACAGCT-3'
		R: 5'- CATGTCCTCCCACAGCTTTC-3'

3. RESULTS

3. Results

3.1. Identification and *in silico* analysis of grapevine miPEP396a (miPEP-MYB5b)

An *in silico* analysis for micropeptide screening allowed the identification of miPEP396a as a candidate miPEP with possible negative regulatory function in the grape berry flavonoid biosynthesis metabolic pathway. Afterwards, to analyze its regulatory function, the identified miPEP was exogenously added to suspension-cultured grape berry cells of the variety Gamay Freaux cv. The miPEP396a is predicted to post-transcriptionally inhibit grapevine transcription factor VvMYB5b in a miRNA-mediated form. Relevant information obtained by the *in silico* analysis regarding miPEP396a (miPEP-MYB5b) including its aminoacidic sequence, attributed name and respective mature miRNA name and miRbase accession number, as well as those of its precursor miRNA (stem-loop), is detailed in **table 2**. Also shown in table 2 is the predicted mode of action of miPEP396a-upregulated miRNA, that is via inhibition by cleavage of its target mRNA (*VvMYB5b*), as well as the biological process in which *VvMYB5b* is involved in, so, that might be negatively regulated by this miPEP.

Table 2. Detailed information about miPEP-MYB5b identified by an *in silico* analysis and selected for the study.

miPEP name	a.a sequence	miRNA name and code	Stem-loop code	Mode of action	Predicted target	Biological process
miPEP396a (miPEP-MYB5b)	MLFHSFLELLF HLPN	miR396a (MIMAT0005724)	MI0006569	Inhibition by cleavage	*VvMYB5b*	Transcription factor positive regulating structural genes of flavonoid pathways

3.2. Effect of the exogenous addition of miPEP396a (miPEP-MYB5b) in the abundance of the corresponding miRNA *Vvi-miR396a*

To assess if the exogenous application of miPEP396a induced the accumulation of its corresponding precursor and mature miRNA (*Vvi-miR396a*) and consequently resulted in a downregulation of its target mRNA, *VvMYB5b*, a real time qPCR analysis was performed. After 5 d of treatment, exogenous miPEP-MYB5b application resulted in a dose-dependent increase in precursor *Vvi-miR396a* transcript levels, as the detected transcript abundance was significantly higher in a

concentration of 0.5 µM of miPEP-MYB5b than in 0.1 µM (**figure 4**). In fact, in control cells, *Vvi-miR396a* transcripts were not detected in our qPCR analysis.

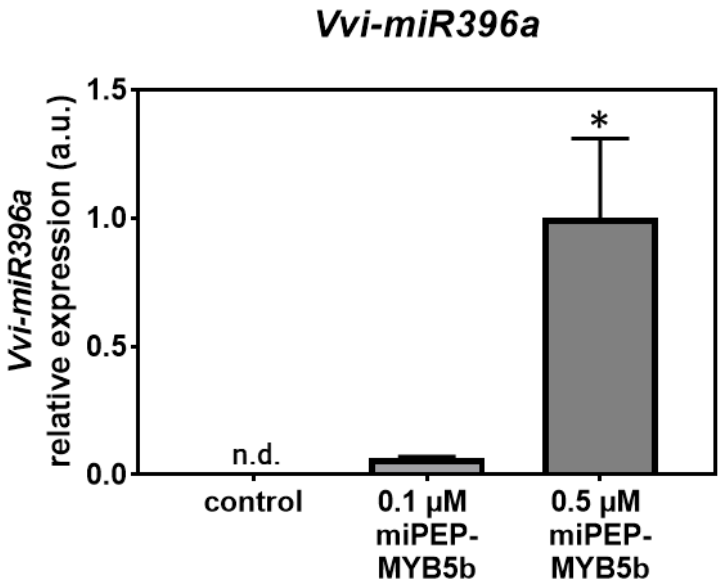

Figure 4. Effect of the exogenous application of miPEP-MYB5b (0.1 µM and 0.5 µM) in the transcript levels of the precursor *Vvi-miR396a* after 5 d of treatment in suspension-cultured grape berry cells and without treatment (Control). Gene expression analysis, by real-time qPCR was normalized with the expression of reference gene *VvACT1* and *VvGAPDH*. Values are the mean ± SEM. Asterisk indicates statistical significance (Student's t-test; *P<0.05; between 0.1 µM and 0.5 µM conditions).

3.3. Effect of the exogenous addition of miPEP-MYB5b on the transcription of *VvMYB5b*

As previously stated, the transcription factor VvMYB5b positively regulates several molecular mechanisms involved in various branches of the flavonoid biosynthetic pathway. The expression of *VvMYB5b* was analyzed in Gamay cells treated with 0.1 µM and 0.5 µM miPEP-MYB5b and compared to control. After 5 d of treatment in grape berry cells, miPEP-MYB5b application resulted in a significant decrease of 50 and 25% in *VvMYB5b* transcript levels for 0.1 µM and 0.5 µM concentrations of miPEP-MYB5b, respectively (**figure 5**).

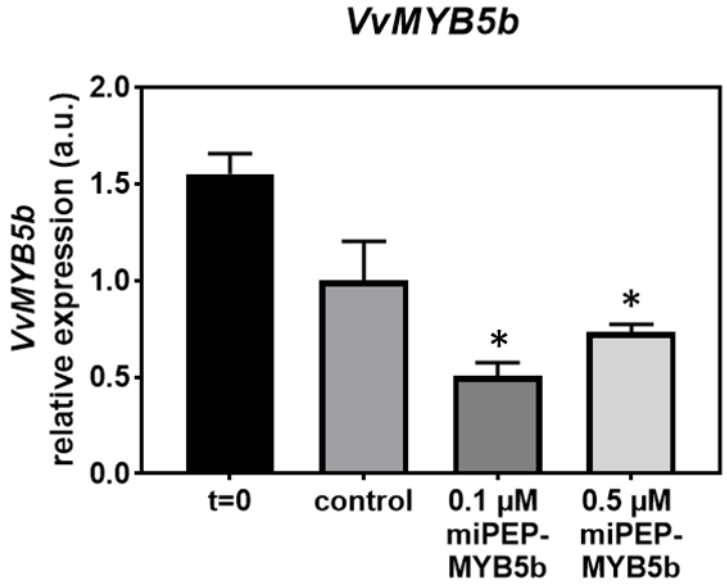

Figure 5. Effect of the exogenous application of miPEP-MYB5b (0.1 µM and 0.5 µM) in the transcript levels of the transcription factor *MYB5b* after 5 d of treatment in suspension-cultured grape berry cells and without treatment (Control). Gene expression analysis, by real-time qPCR was normalized with the expression of reference gene *VvACT1* and *VvGAPDH*. Values are the mean ± SEM. Asterisk indicates statistical significance (Student's t-test; *P<0.05; between 0.1 µM and 0.5 µM conditions).

3.4. The effect of the exogenous addition of miPEP-MYB5b effect on the concentration of secondary metabolites in grape berry cells

3.4.1. Flavonoids

After 5 d of miPEP-MYB5b treatment on suspension-cultured Gamay cells, plant molecular and biochemical approaches were conducted to assess the effect of this miPEP that putatively inhibits transcription factor VvMYB5b. An emphasis was given to the phenylpropanoid, flavonoid and stilbene pathway, the biosynthetic routes that produce several bioactive compounds that confer quality-traits to grape berry. Flavonoids include flavones, flavonols, flavanones, flavan-3-ols and anthocyanins and its intracellular concentration can fluctuate due to external stressors and influence the overall quality of the berry. Their quantification was an important goal of this study, as the pathway that leads to their biosynthesis competes directly for substrate with the stilbene pathway, thus a putative inhibition of flavonoid synthesis mediated by this miPEP may lead to higher substrate availability to feed stilbene synthesis.

As shown in **figure 6,** the intracellular flavonoid concentration decreased about 25% when both concentrations of miPEP were tested, compared to control, reaching nearly 1100 µg g DW^{-1}.

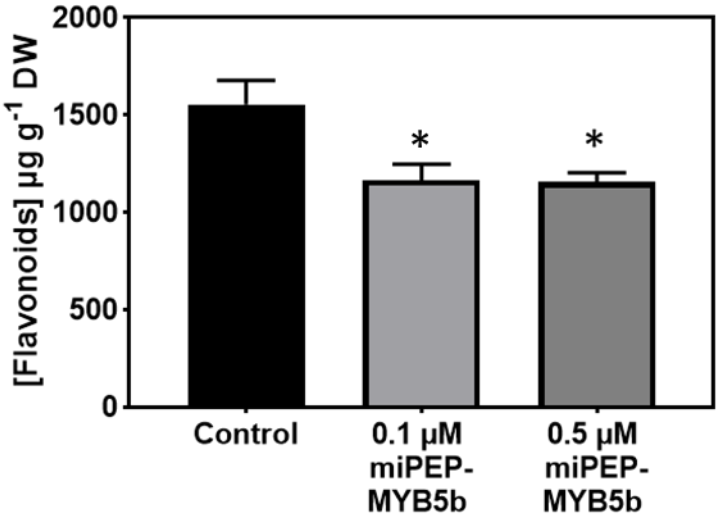

Figure 6. Effect of the exogenous application of miPEP-MYB5b (0.1 µM and 0.5 µM) on total flavonoid content after 5 d of treatment in suspension-cultured grape berry cells. Flavonoid concentration is represented as µg of quercetin equivalents per g of dry weight (DW). Asterisk indicates statistical significance (Student's t-test; *P<0.05).

Intracellular quantities of some paradigmatic flavonoids were also evaluated by HPLC. Both the flavan-3-ol epicatechin and quercetin-3-glucoside content decreased in cells treated with miPEP-MYB5b (**figure 7**). When compared to the control, after 5 d of treatment, both concentrations of miPEP revealed a similar effect in decreasing epicatechin content. However, miPEP-MYB5b at concentration of 0.5 µM had a higher effect in decreasing quercetin -3-glucoside content than 0.1 µM.

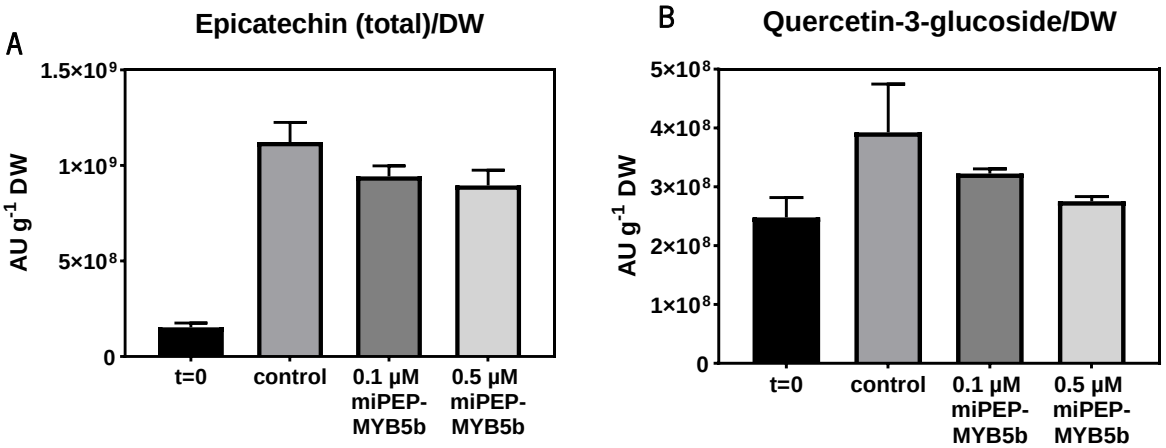

Figure 7. Effect of the exogenous application of miPEP-MYB5b (0.1 µM and 0.5 µM) on epicatechin (A) and quercetin-3-glucoside (B) content after 5 d of treatment in suspension-cultured grape berry cells. Flavonoid concentration is represented as arbitrary units per g of dry weight (DW).

3.4.2. Anthocyanins

Intracellular and extracellular anthocyanin concentration determination was also performed. As shown in **figure 8A**, both concentrations of miPEP decreased anthocyanin intracellular concentration in

grape berry cells. After 5 d of treatment, the concentration of 0.1 µM of miPEP-MYB5b had a higher inhibitory effect, lowering the anthocyanin concentration down to 3.75 mg of cyanidin-3-glucoside equivalents per g of DW, when compared to 10 mg per g of DW in control cells, while cells treated with 0.5 µM miPEP-MYB5b reached 5.5 mg g DW^{-1}. However, this concentration had a higher effect in decreasing extracellular anthocyanin concentration (**figure 8B**) after 5 d of treatment, while 0.1 µM miPEP led to an increase of anthocyanin concentration when comparing to the control. Still, the extracellular anthocyanin concentration was rather low in all experimental conditions, never exceeding 4 mg L^{-1}.

Furthermore, we also detected three anthocyanins in HPLC-DAD analysis based on their maximum absorbance wavelength, peak form and retention time, all typical of anthocyanins (**figure 9**). However, we were not able to specify the anthocyanins. Both concentrations of miPEP-MYB5b slightly decreased the content of the third anthocyanin (**figure 9C**).

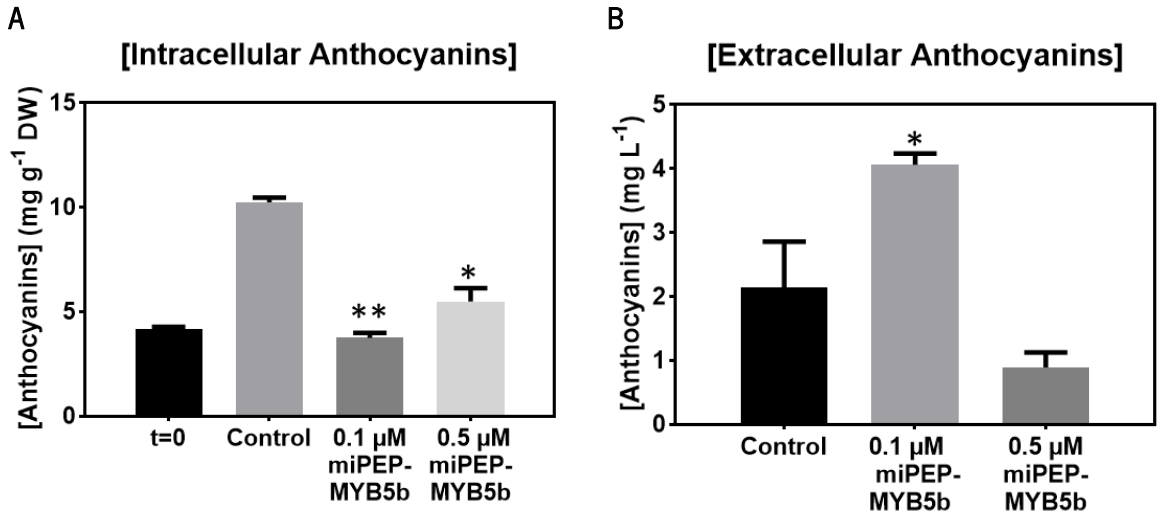

Figure 8. Effect of the exogenous application of miPEP-MYB5b (0.1 µM and 0.5 µM) on intracellular (A) and extracellular (B) anthocyanin concentration after 5 d of treatment in suspension-cultured grape berry cells. Anthocyanin concentration is represented as mg cyanidin 3-glucoside (C-3-G) equivalents per g of dry weight (DW).

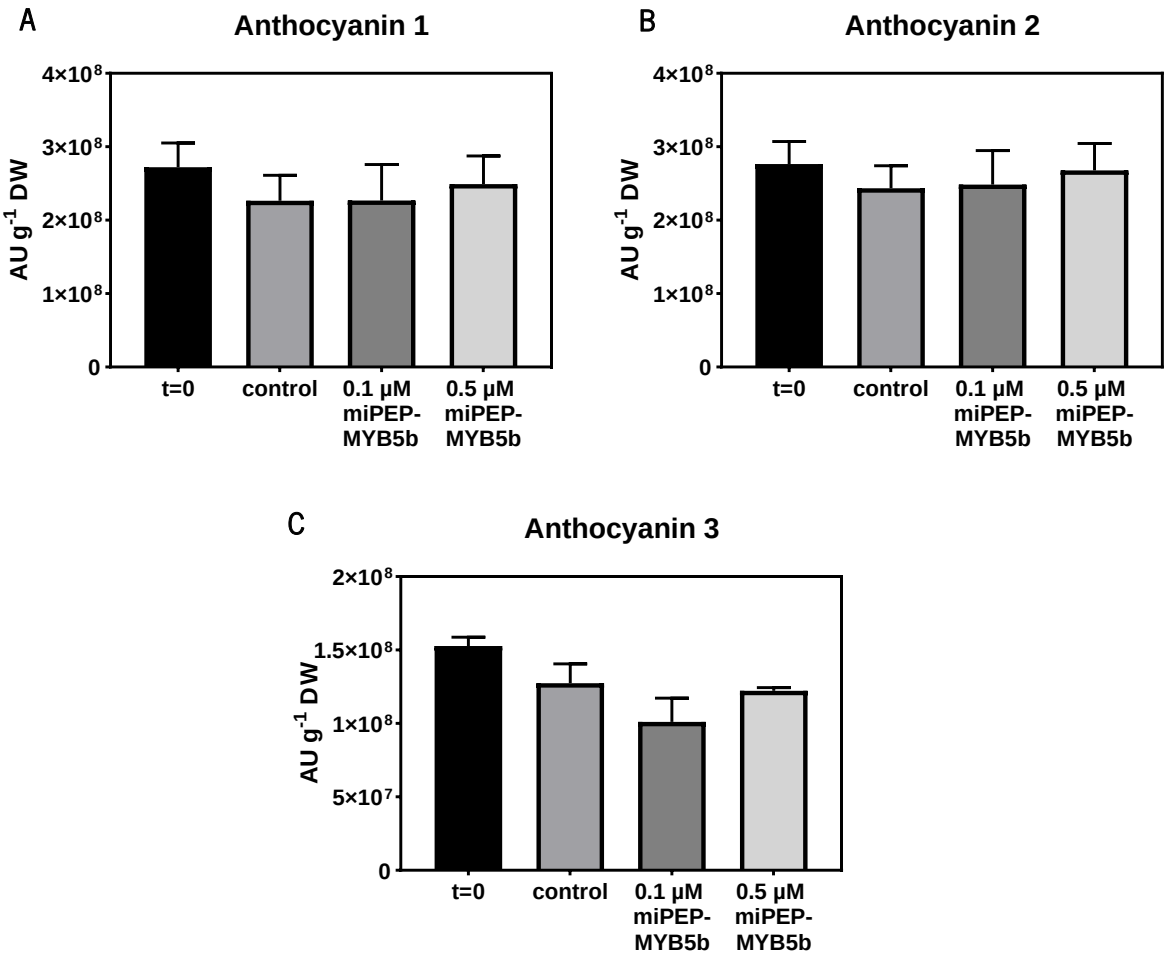

Figure 9. Effect of the exogenous application of miPEP-MYB5b (0.1 µM and 0.5 µM) on anthocyanin 1 (A) anthocyanin 2 (B) and anthocyanin 3 (C) content after 5 d of treatment in suspension-cultured grape berry cells. Anthocyanin content is represented as arbitrary units per g of dry weight (DW).

3.4.3. Stilbenes

In this study we wanted to address the hypothesis that miPEP-MYB5b–mediated inhibition of general genes of the flavonoid pathway stimulate stilbene synthesis. Thus, the assessment of intracellular quantities of stilbenes was crucial. HPLC-DAD analysis allowed to detect a viniferin and *trans*-piceid in control and grape berry treated cells (**figure 10)**. Intracellular *trans*-piceid content had no significant differences between control and both concentrations of miPEP (**figure 10A)**. Contrarily, both concentrations of miPEP stimulated intracellular viniferin content, when compared to control (**figure 10B)**, with the 0.1 µM concentration of miPEP-MYB5b being more effective in increasing viniferin content.

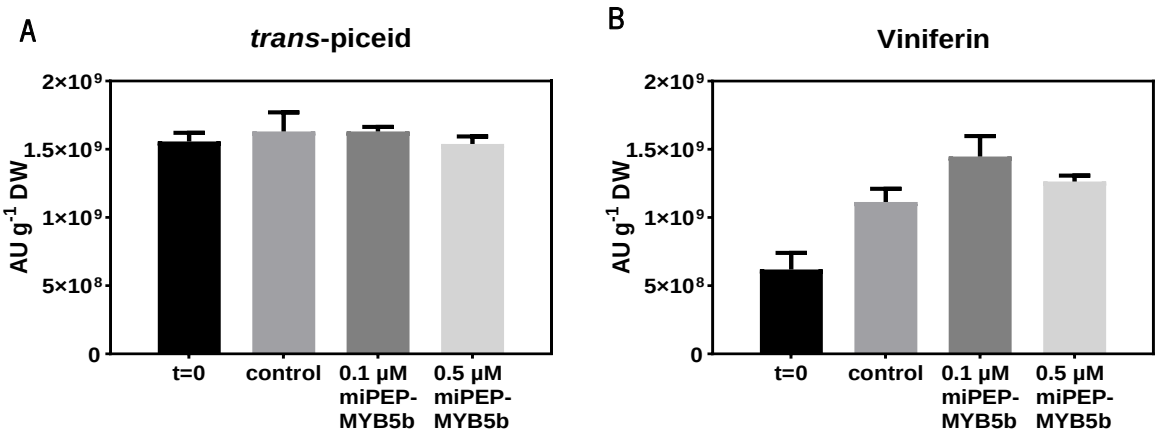

Figure 10. Effect of the exogenous application of miPEP-MYB5b (0.1 µM and 0.5 µM) on *trans*-piceid (A) and a viniferin (B) content after 5 d of treatment in suspension-cultured grape berry cells. *Trans*-piceid and viniferin content is represented as arbitrary units per g of dry weight (DW).

3.4.4. Total phenolics concentration

Both intracellular and extracellular concentration of phenolics were also determined in miPEP-MYB5b treated and control grape berry cells to evaluate its effect in general synthesis of phenolic compounds. As displayed in **figure 11**, both concentrations of miPEP decreased intracellular phenolic compounds concentration, reaching about 75 mg g DW^{-1} in cells subjected to 0.1 µM miPEP-MYB5b (**figure 11A**). The concentration of extracellular phenolics was significantly reduced by a treatment with 0.5 µM miPEP-MYB5b, reaching about 80 mg g DW^{-1}, when compared to more than 200 mg g DW^{-1} in the control (**figure 11B**).

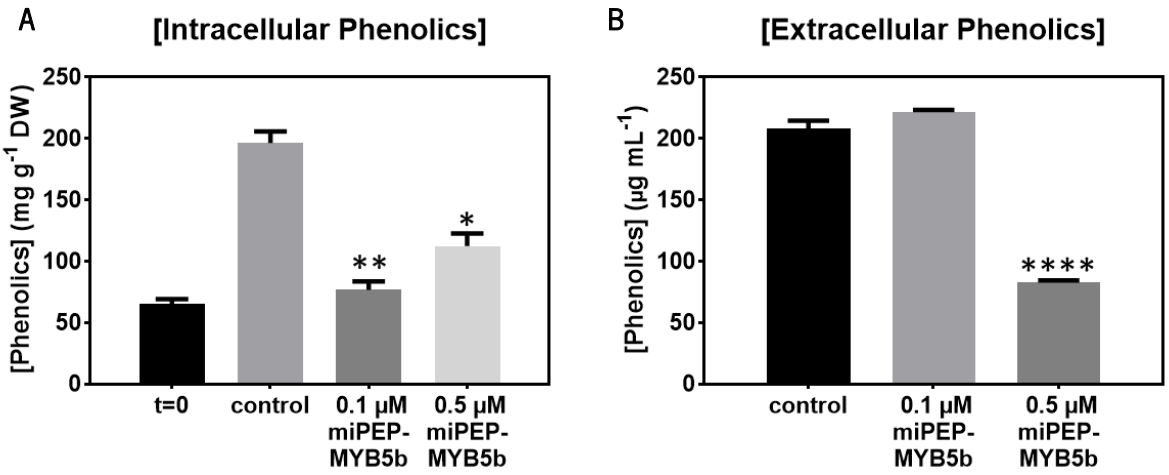

Figure 11. Effect of the exogenous application of miPEP-MYB5b (0.1 µM and 0.5 µM) on intracellular (A) and extracellular (B) total phenolics concentration after 5 d of treatment in suspension-cultured grape berry cells. Total phenolics concentration is represented as µg of gallic acid equivalents (GAE) per mg of dry weight (DW). Asterisks indicate statistical significance (Student's t-test; * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$).

3.5. Transcriptional changes in secondary metabolic pathways induced by the exogenous application of miPEP-MYB5b

3.5.1. Flavonoids-biosynthetic pathway

Transcriptional changes in molecular mechanisms involved in flavonoid synthesis induced by miPEP-MYB5b were also evaluated by real time qPCR in Gamay cells. The flavonoids pathway, after initial common steps, branches into pathways leading to the synthesis of proanthocyanidins and flavan-3-ols branch, flavonols, and anthocyanins.

The enzyme dihydroflavonol 4-reductase (DFR) converts dihydroflavonols into leucoanthocyanidins, which are the immediate precursors of both proanthocyanidins and anthocyanins. On the other hand, leucoanthocyanidins can also be catalyzed by the enzyme leucoanthocyanidin dioxygenase (LDOX, also named anthocyanidin synthase (ANS)) for the synthesis of proanthocyanidins. The expression levels of *VvDFR* reduced in cells treated with miPEP, when compared to control (**figure 12A**). In cells treated with 0.1 µM miPEP, the expression levels decreased 10% but in cells treated with 0.5 µM they decreased almost 50%. Moreover, transcriptional analyses revealed that 0.1 µM miPEP led to a very significant 2-fold increase in the expression of *VvLDOX*, when compared to the control (**figure 12B**). However, 0.5 µM miPEP only increased *VvLDOX* transcripts by 12%.

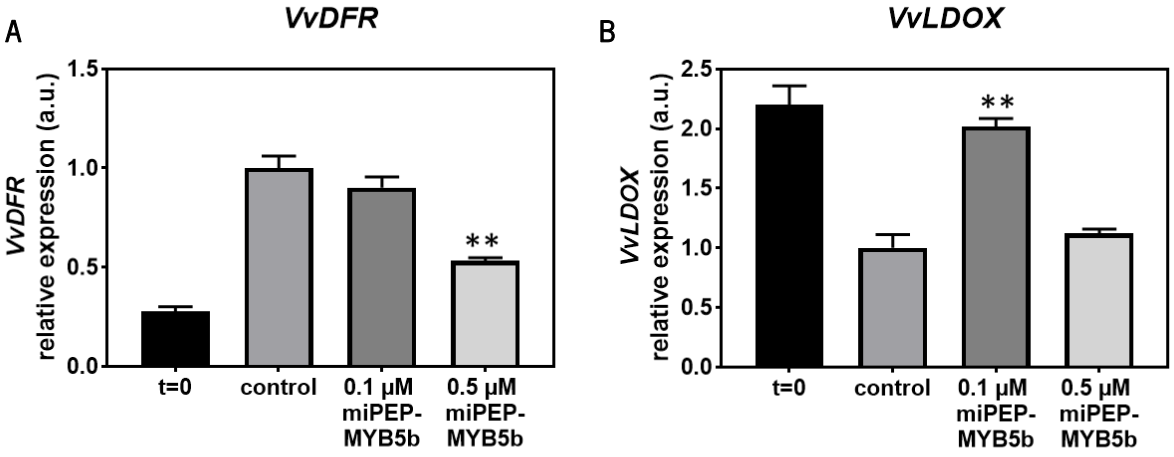

Figure 12. Effect of the exogenous application of miPEP-MYB5b (0.1 µM and 0.5 µM) in the transcript levels of *VvDFR* (A) and *VvLDOX* (B) after 5 d of treatment in suspension-cultured grape berry cells and without treatment (Control). Gene expression analysis, by real-time qPCR was normalized with the expression of reference gene *VvACT1* and *VvGAPDH*. Values are the mean ± SEM. Asterisks indicate statistical significance (Student's t-test; ** $P < 0.01$).

3.5.1.1. Flavonols

Chalcone synthase (CHS) is the first enzyme responsible for flavonoid biosynthesis, redirecting the substrate 4-coumaroyl-CoA to the flavonoid branch. CHS competes directly with stilbene synthase (STS), the key enzyme that redirects the carbon flow from the phenylpropanoid pathway into the stilbene pathway. Following the flavonoid pathway, the chalcone isomerase (CHI) is an enzyme responsible for the isomerisation of chalcone to naringenin, then flavonoid 3',5'-hydroxylases (F3'5'Hs) control the synthesis of delphinidin, the precursor of blue anthocyanins and, lastly, the flavonol synthase (FLS) redirects the carbon flow to the flavonol branch, by converting dihydroflavonols to flavonols such as kaempferol, myricetin and quercetin.

Transcriptional analyzes of *VvCHS1* revealed that 0.1 µM miPEP induced no significant changes when compared to control (**figure 13A**). On the other hand, 0.5 µM miPEP led to a decrease of 45%. The expression levels of *VvCHS3* decreased about 5% in cells treated with 0.1 µM miPEP but increased almost 65% in cells treated with 0.5 µM miPEP, when compared to control (**figure 13B**).

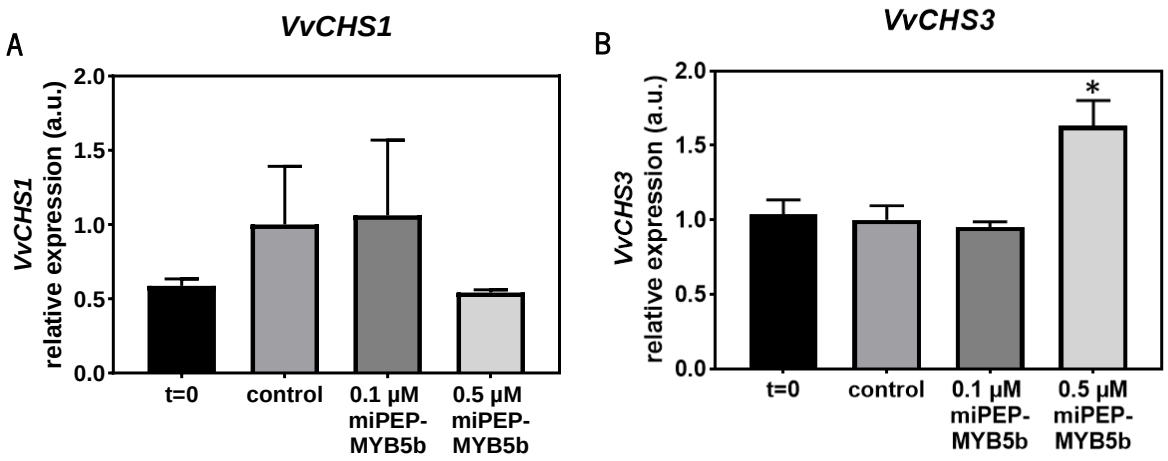

Figure 13. Effect of the exogenous application of miPEP-MYB5b (0.1 µM and 0.5 µM) in the transcript levels of *VvCHS1* (A) and *VvCHS3* (B) after 5 d of treatment in suspension-cultured grape berry cells and without treatment (Control). Gene expression analysis, by real-time qPCR was normalized with the expression of reference gene *VvACT1* and *VvGAPDH*. Values are the mean ± SEM. Asterisks indicate statistical significance (Student's t-test; * $P < 0.05$).

Furthermore, the expression levels of *VvCHI* decreased by ca. 30% in cells treated with both concentrations of miPEP, when compared to control (**figure 14A**). Contrarily, *VvF3'5'H* expression levels increased 16% and 63% in cells treated with 0.1 µM and 0.5 µM miPEP, respectively, when compared to control (**figure 14B**). Lastly, the expression levels of *VvFLS1* had no significant changes in cells treated with 0.1 µM miPEP, when compared to control (**figure 14C**). However, in cells treated with 0.5 µM miPEP, the expression levels decreased 40%.

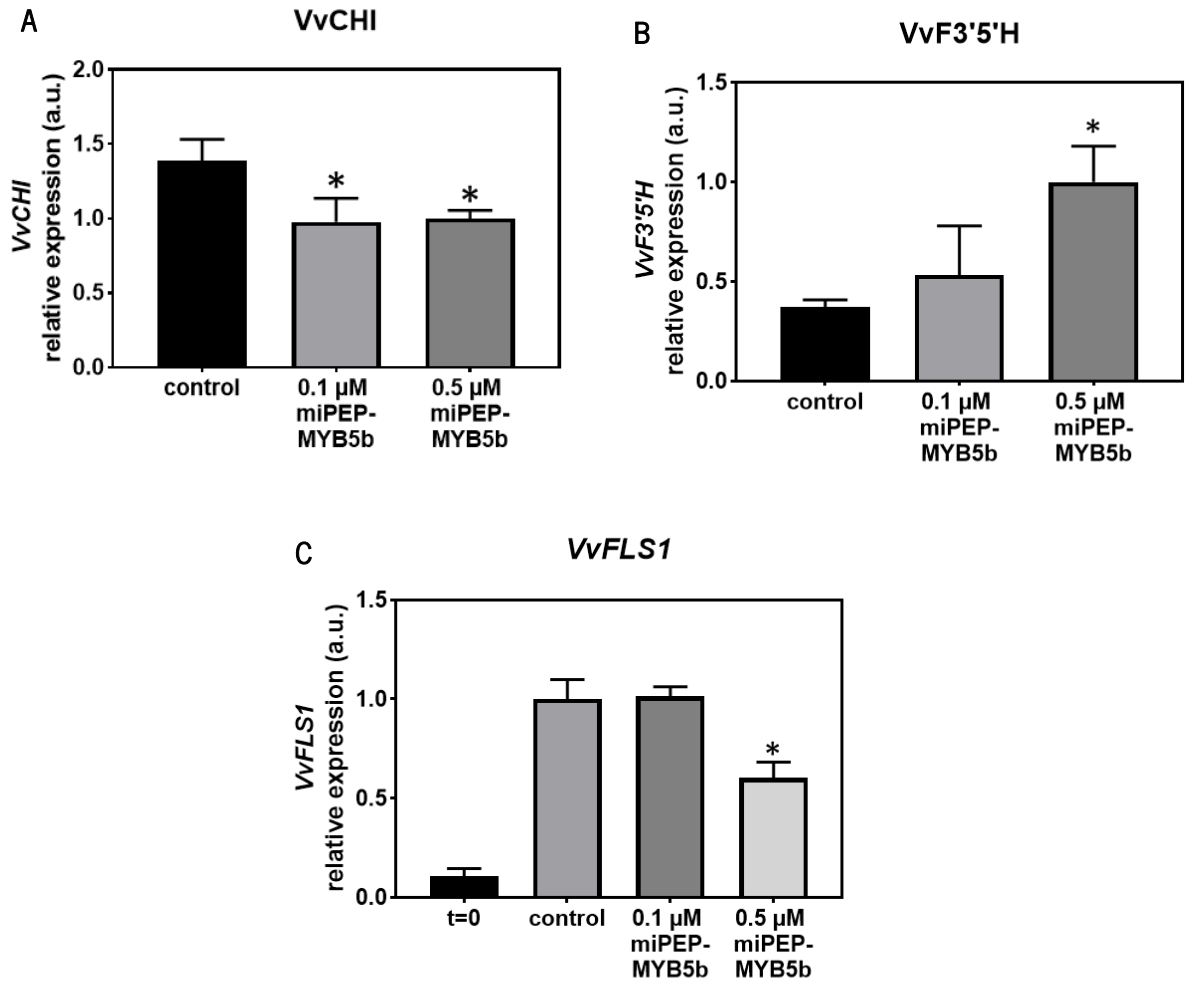

Figure 14. Effect of the exogenous application of miPEP-MYB5b (0.1 µM and 0.5 µM) in the transcript levels of *VvCHI* (A), *VvF3'5'H* (B) and *VvFLS1* (C) after 5 d of treatment in suspension-cultured grape berry cells and without treatment (Control). Gene expression analysis, by real-time qPCR was normalized with the expression of reference gene *VvACT1* and *VvGAPDH*. Values are the mean ± SEM. Asterisks indicate statistical significance (Student's t-test; * $P < 0.05$).

3.5.1.2. Proanthocyanidins and flavan-3-ols

As previously mentioned, leucoanthocyanidins can be catalyzed by LDOX, continuing the flavonoid pathway and resulting in the formation of proanthocyanidins or anthocyanidins. Anthocyanidins can either result in the formation of anthocyanins through the action of UDP-glucose:flavonol 3-O-D-glucosyltransferase (UFGT) or of the flavan-3-ol epicatechin in a reaction catalyzed by anthocyanidin reductase (ANR). On the other hand, dihydroflavonols can also be reduced into leucoanthocyanidin, a precursor of flavan-3-ols such as catechins, by the action of leucoanthocyanidin reductase (LAR) and produce proanthocyanidins.

The expression levels of *VvLAR1* reduced 45% in cells treated with 0.1 µM miPEP and almost 75% in cells treated with 0.5 µM miPEP, when compared to control (**figure 15A**). However, the

expression levels of *VvLAR2* reduced by almost 40% in cells treated with 0.1 µM concentration of miPEP and almost 20% in cells treated with 0.5 µM miPEP, when compared to control (**figure 15B**).

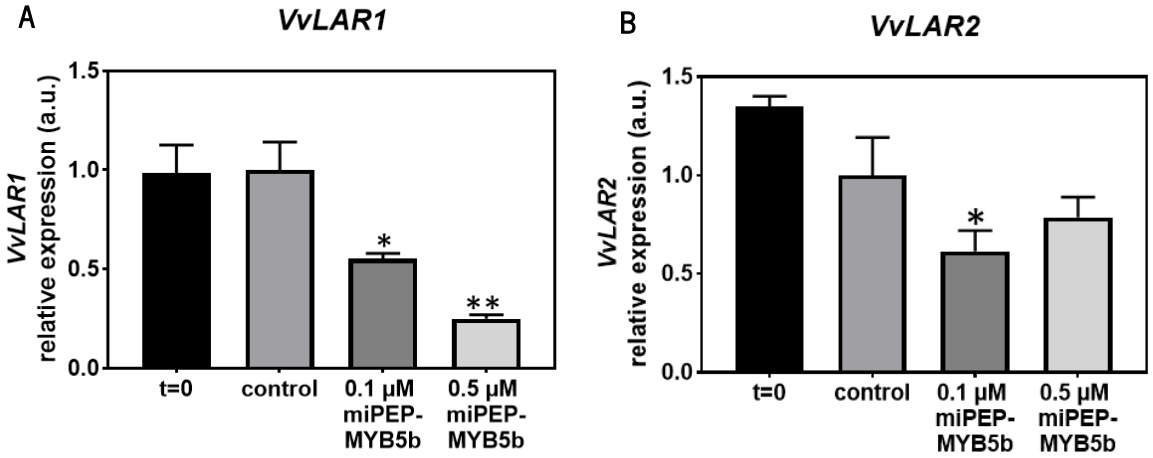

Figure 15. Effect of the exogenous application of miPEP-MYB5b (0.1 µM and 0.5 µM) in the transcript levels of *VvLAR1* (A) and *VvLA2* (B) after 5 d of treatment in suspension-cultured grape berry cells and without treatment (Control). Gene expression analysis, by real-time qPCR was normalized with the expression of reference gene *VvACT1* and *VvGAPDH*. Values are the mean ± SEM. Asterisks indicate statistical significance (Student's t-test; * $P < 0.05$, ** $P < 0.01$).

Furthermore, transcriptional analysis of *VvANR* revealed that 0.1 µM miPEP had no significant effect but 0.5 µM miPEP led to a decrease of almost 45%, when compared to control (**figure 16**).

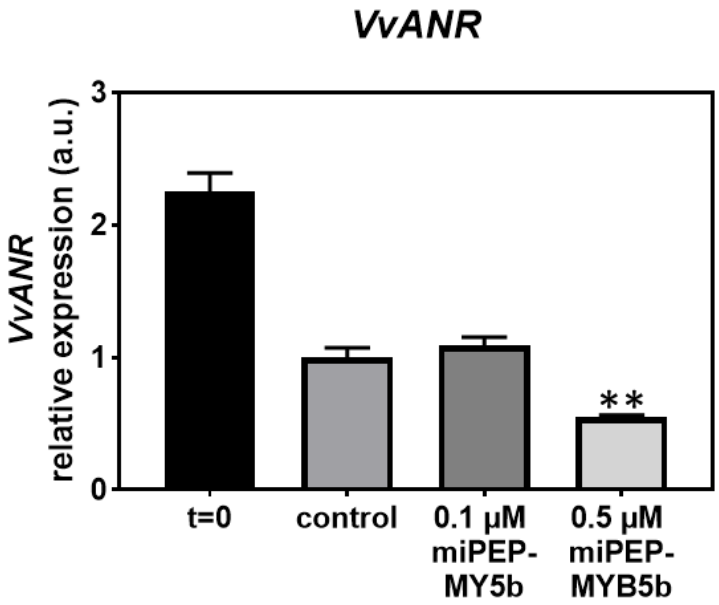

Figure 16. Effect of the exogenous application of miPEP-MYB5b (0.1 µM and 0.5 µM) in the transcript levels of *VvANR* after 5 d of treatment in suspension-cultured grape berry cells and without treatment (Control). Gene expression analysis, by real-time qPCR was normalized with the expression of reference gene *VvACT1* and *VvGAPDH*. Values are the mean ± SEM. Asterisks indicate statistical significance (Student's t-test; ** $P < 0.01$).

3.5.1.3. Anthocyanins

UDP-glucose flavonoid 3-O-glucosyltransferase (UFGT) is the final enzyme in the flavonoid biosynthetic pathway in the branch of anthocyanin synthesis, catalyzing the conversion of anthocyanidins, through a glycosylation reaction, into anthocyanins. The transcriptional analysis of *VvUFGT1* showed no significant changes in cells treated with 0.1 µM miPEP, when compared to control. However, in cells treated with 0.5 µM concentration of miPEP, the expression levels decreased about 10% in a non-statistically significant way (**figure 17**).

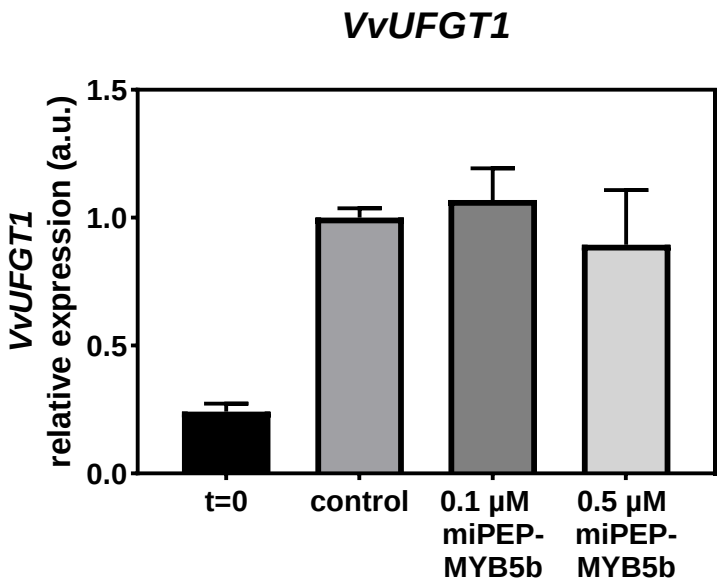

Figure 17. Effect of the exogenous application of miPEP-MYB5b (0.1 µM and 0.5 µM) in the transcript levels of *VvUFGT1* after 5 d of treatment in suspension-cultured grape berry cells and without treatment (Control). Gene expression analysis, by real-time qPCR was normalized with the expression of reference gene *VvACT1* and *VvGAPDH*. Values are the mean ± SEM.

3.5.2. Anthocyanin stabilization, transport to the vacuole and degradation

Anthocyanins are accumulated in the vacuole of plant cells. Two types of anthocyanin tonoplast transporters have been identified in grapevine: primary transporters from the ATP-binding cassette (ABC) family, such as the VvABCC1 who requires the presence of reduced glutathione (GSH) to properly transport anthocyanins into the vacuole; and secondary transporters like VvMATE1 (anthoMATE) of the multidrug and toxic extrusion family that use the H^+ gradient to transport mostly acylated anthocyanins. Also critical for anthocyanin stabilization and transport are the glutathione S-transferases, as grapevine's VvGST4, to promote anthocyanin S-conjugation with reduced glutathione for anthocyanin-stabilization purposes.

Transcriptional analysis of *VvABCC1* revealed that cells treated with miPEP had increased transcript levels, when compared do control (**figure 18A**). In cells treated with 0.1 µM miPEP, the expression levels increased 35% and in cells treated with 0.5 µM the expression levels had a very significant increase of 3.6-fold. On the other hand, the expression levels of *VvMATE1* decreased 8% and 47% in cells treated with 0.1 µM and 0.5 µM, respectively, when compared to control (**figure 18B**). Additionally, the expression levels of *VvGST4* increased almost 20% but decreased almost 50% in cells treated with 0.1 µM and 0.5 µM concentrations, respectively, when compared to control (**figure 18C**).

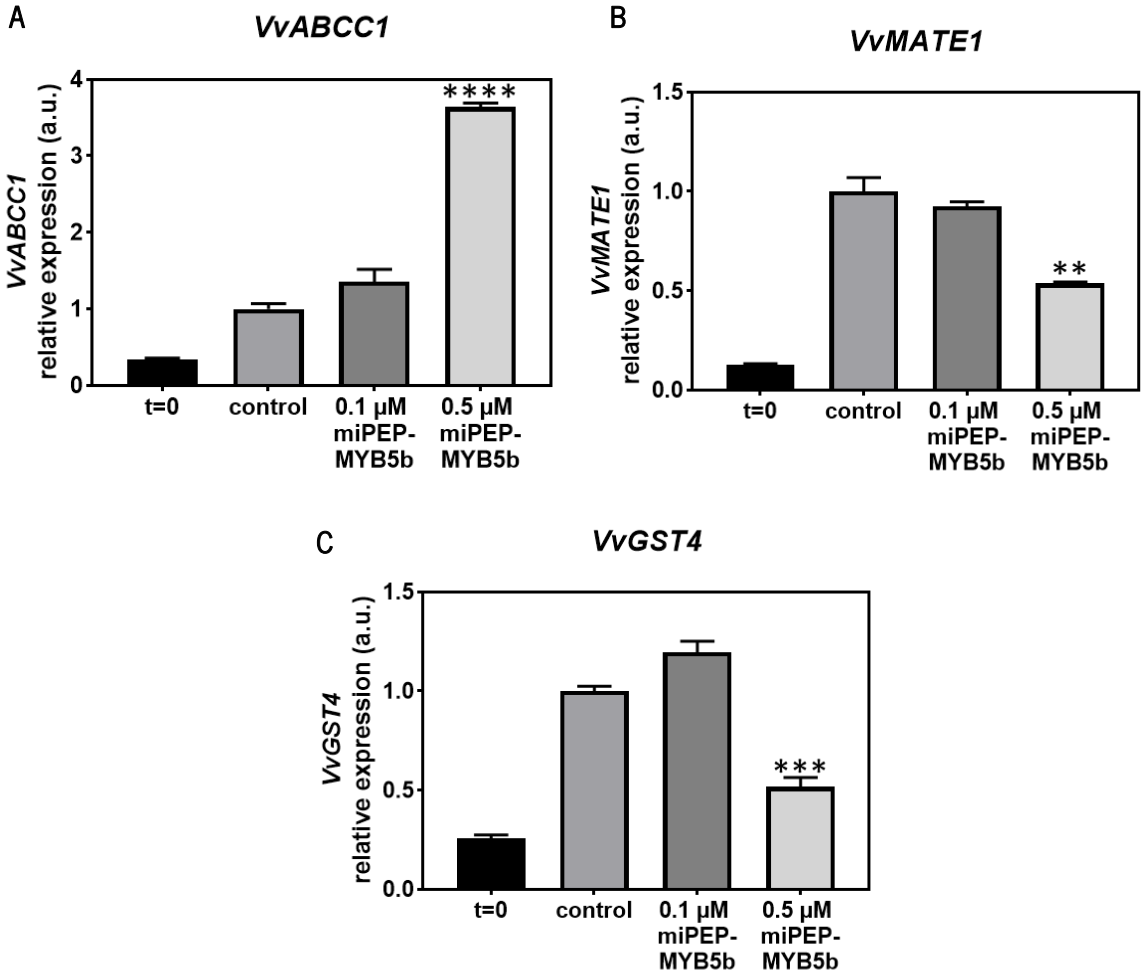

Figure 18. Effect of the exogenous application of miPEP-MYB5b (0.1 µM and 0.5 µM) in the transcript levels of *VvABCC1* (A), *VvMATE1* (B) and (C) *VvGST4* after 5 d of treatment in suspension-cultured grape berry cells and without treatment (Control). Gene expression analysis, by real-time qPCR was normalized with the expression of reference gene *VvACT1* and *VvGAPDH*. Values are the mean ± SEM. Asterisks indicate statistical significance (Student's t-test; ** $P < 0.01$, *** $P< 0.001$, **** $P< 0.0001$).

In this study, we also analyzed miPEP-MYB5b effect on anthocyanin degradation following a transcriptional analysis of the peroxidase *VvPRX31*. The expression levels of *VvPRX31* significantly decreased 88% in cells treated with 0.1 µM miPEP but increased 60% in cells treated with 0.5 µM, when compared to control (**figure 19**).

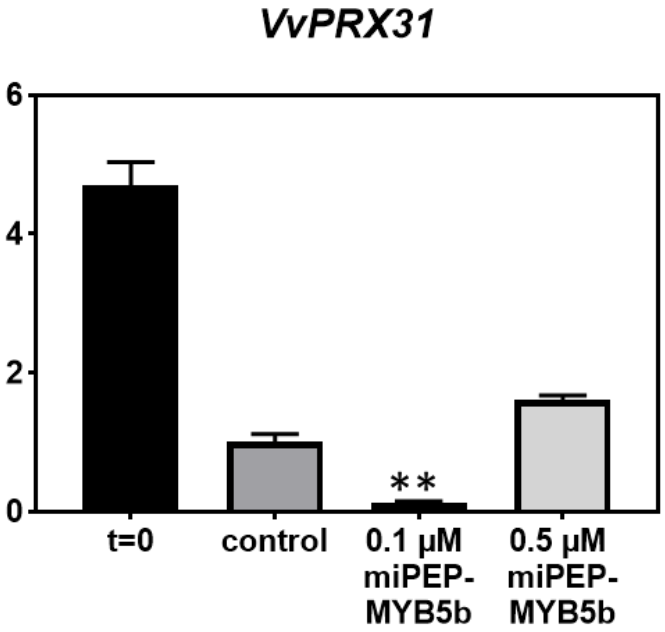

Figure 19. Effect of the exogenous application of miPEP-MYB5b (0.1 µM and 0.5 µM) in the transcript levels of *VvPRX31* after 5 d of treatment in suspension-cultured grape berry cells and without treatment (Control). Gene expression analysis, by real-time qPCR was normalized with the expression of reference gene *VvACT1* and *VvGAPDH*. Values are the mean ± SEM. Asterisks indicate statistical significance (Student's t-test; ** $P < 0.01$).

3.5.3. Transcriptional changes in stilbene pathway

The stilbene pathway is a branch of the general phenylpropanoid pathway, where stilbene synthase (STS) encodes for the first key enzyme that redirects the carbon flow from phenylpropanoids to the stilbene pathway branch. This branch competes with chalcone synthase (CHS), the first enzyme in the flavonoid pathway, for the end-product of the phenylpropanoid pathway, the substrate 4-coumaroyl-CoA.

Transcriptional analysis of *VvSTS1* revealed that cells treated with 0.1 µM miPEP-MYB5b presented a very significant increase of nearly 2-fold but in cells treated with 0.5 µM miPEP the *VvSTS1* expression levels had no significant changes, when compared to control (**figure 20**).

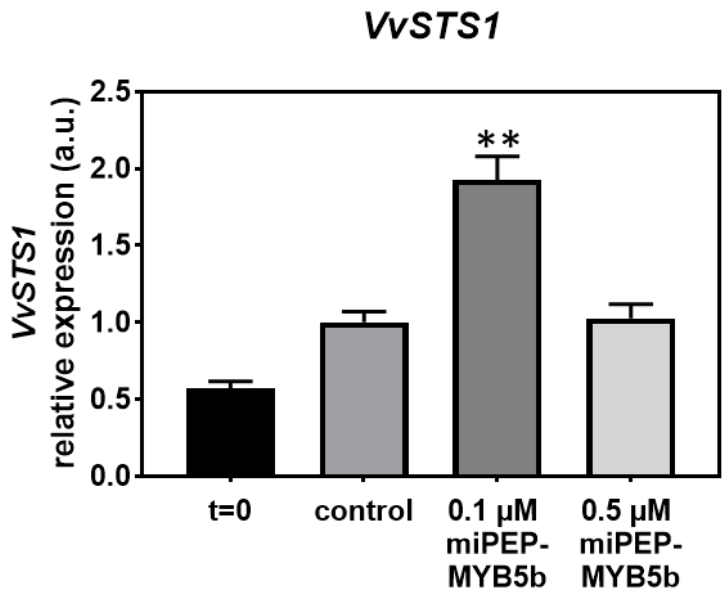

Figure 20. Effect of the exogenous application of miPEP-MYB5b (0.1 µM and 0.5 µM) in the transcript levels of *VvSTS1* after 5 d of treatment in suspension-cultured grape berry cells and without treatment (Control). Gene expression analysis, by real-time qPCR was normalized with the expression of reference gene *VvACT1* and *VvGAPDH*. Values are the mean ± SEM. Asterisks indicate statistical significance (Student's t-test; ** $P < 0.01$).

3.5.4. Transcriptional changes in phenylpropanoid pathway

The phenylpropanoid pathway starts with the conversion of phenylalanine to 4-couramoyl-CoA by phenylalanine ammonia-lyase (PAL), the key enzyme catalyzing the first step in the synthesis of all phenylpropanoids. Then, the enzyme cinnamate-4-hydroxylase (C4H) converts cinnamate to p-coumaric acid by hydroxylation. The esterification of p-coumaric by the enzyme CoA-ligase (4CL) results in the biosynthesis of p-coumaroyl-CoA, a molecule that is the substrate of two key enzymes in parallel and competing pathways: chalcone synthase (CHS) and stilbene synthase (STS).

Transcriptional analysis of *VvPAL1* revealed that cells treated with 0.1 µM concentration of miPEP had almost a 3-fold increase while cells treated with 0.5 µM concentration of miPEP only increased 44%, when compared to control (**figure 21A**). Additionally, the expression levels of *VvC4H* had a 2-fold increase in cells treated with 0.1 µM concentration of miPEP but only increased about 80% in cells treated with 0.5 µM concentration of miPEP, when compared to control (**figure 21B**).

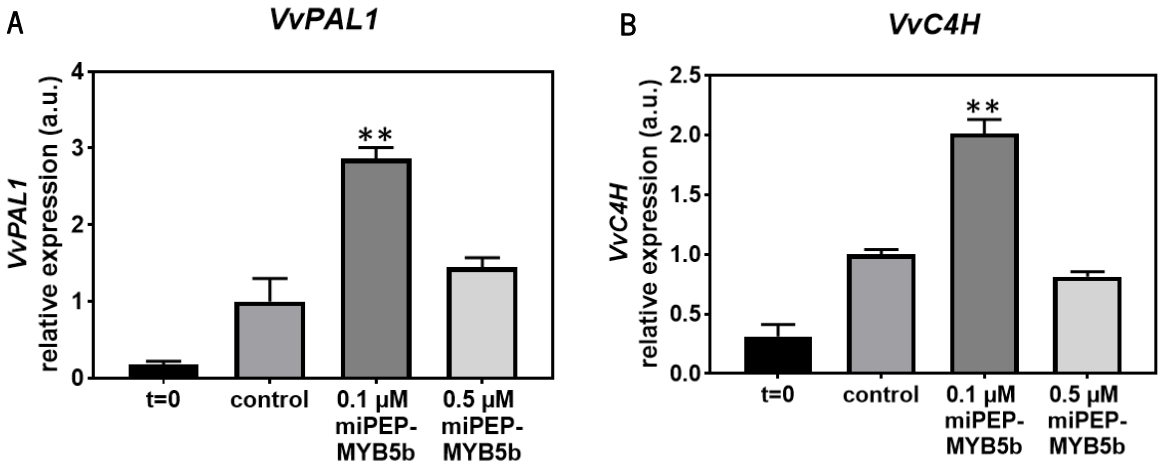

Figure 21. Effect of the exogenous application of miPEP-MYB5b (0.1 µM and 0.5 µM) in the transcript levels of *VvPAL1* (A) and *VvC4H* (B) after 5 d of treatment in suspension-cultured grape berry cells and without treatment (Control). Gene expression analysis, by real-time qPCR was normalized with the expression of reference gene *VvACT1* and *VvGAPDH*. Values are the mean ± SEM. Asterisks indicate statistical significance (Student's t-test; ** $P < 0.01$).

3.5.5. Transcriptional changes in norisoprenoid pathway

Norisoprenoids are a group of compounds derived from the degradation of carotenoids, a group of tetraterpenoid pigments extensively existing in plants. Norisoprenoids, with 13 carbon atoms, are among numerous well-known scent compounds with extremely low sensory thresholds and are also important sources of grape-derived flavors in wines. Plant isoprenoids are produced through the distinct but cross-talking cytosolic mevalonate (MVA) and plastidial 2-C-methyl-d-erythritol 4-phosphate (MEP) routes. In this study, we analyzed the expression levels of *VvCCD4b*, a carotenoid cleavage dioxygenase. Transcriptional analysis of *VvCCD4b* revealed that 0.1 µM miPEP had no significant changes but 0.5 µM miPEP had an increase of almost 30%, when compared to control, though not statistically significant (**figure 22**).

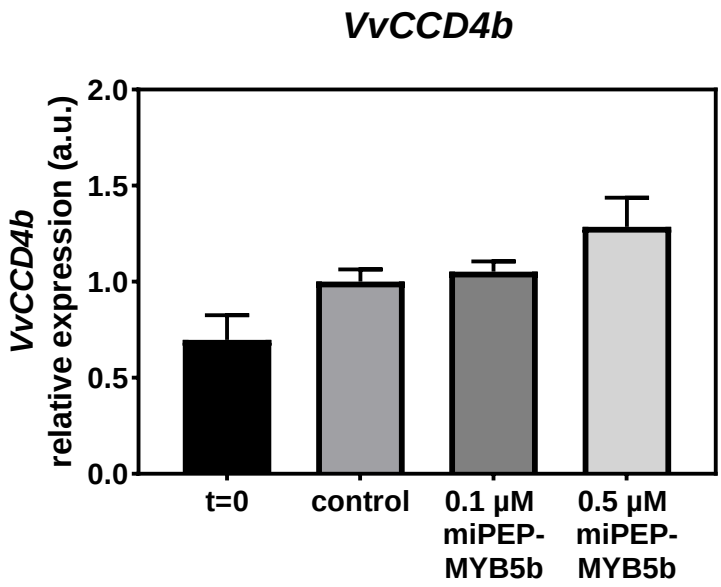

Figure 22. Effect of the exogenous application of miPEP-MYB5b (0.1 µM and 0.5 µM) in the transcript levels of *VvCCD4b* after 5 d of treatment in suspension-cultured grape berry cells and without treatment (Control). Gene expression analysis, by real-time qPCR was normalized with the expression of reference gene *VvACT1* and *VvGAPDH*. Values are the mean ± SEM.

3.5.6. Transcriptional changes in other transcription factors regulating flavonoids synthesis

The grapevine transcription factor MYBPA1 is significantly involved in the transcriptional activation of molecular mechanisms, such as *VvANR* and *VvLAR1*, leading to proanthocyanidin formation. Anthocyanin synthesis is specifically regulated by transcriptional regulators as VvMYBA1 or VvMYBA2, through activation of UFGT transcription. It is also described that VvMYBA1 is involved in the activation of genes present in the anthocyanin transport process.

In cells treated with 0.1 µM miPEP-MYB5b, the expression levels of *VvMYBPA1* increased 33% but in cells treated with 0.5 µM miPEP, the expression levels decreased 40%, when compared to control (**figure 23A**). Contrarily, in cells treated with 0.1 µM miPEP, the expression levels of *VvMYBA1* decreased 62% but in cells treated with 0.5 µM miPEP the expression levels had a 3.2-fold increase, when compared to control (**figure 23B**).

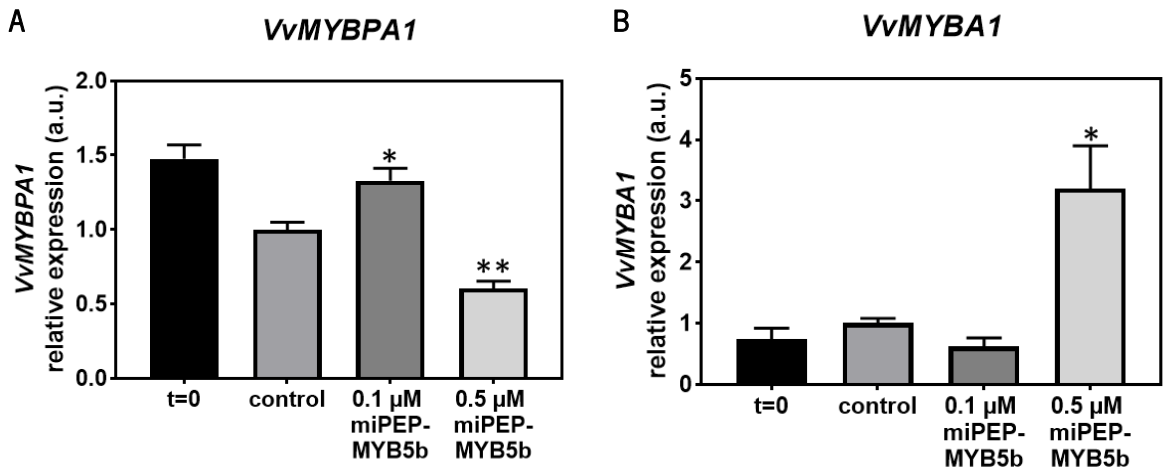

Figure 23. Effect of the exogenous application of miPEP-MYB5b (0.1 µM and 0.5 µM) in the transcript levels of *VvMYBPA1* (A) and *VvMYBA1* (B) after 5 d of treatment in suspension-cultured grape berry cells and without treatment (Control). Gene expression analysis, by real-time qPCR was normalized with the expression of reference gene *VvACT1* and *VvGAPDH*. Values are the mean ± SEM. Asterisks indicate statistical significance (Student's t-test; * $P < 0.05$, ** $P < 0.01$).

3.6. Maximization of stilbene production in Gamay cells by the exogenous application of Methyl Jasmonate, beta-methylcyclodextrin and miPEP-MYB5b

Exogenous application of methyl jasmonate (MeJa), beta-methylciclodextrin (beta-MCD) and miPEP-MYB5b was also performed to study if this combination of elicitors could be a strategy to massively increase stilbene biosynthesis in grape berry cells, and also if the addition of miPEP-MYB5b could exacerbate a theoretically increase caused by the combination of MeJa and beta-MCD only.

HPLC analysis revealed that intracellular *trans*-piceid and a viniferin were detected but in lower concentrations in cells treated with the elicitors MeJa and beta-MCD and with the triple combination (**figure 24A and C**). However, extracellular *trans*-piceid and viniferin were highly detected in the culture media of cells subjected to those conditions, contrarily to the medium of control cells, where neither of the stilbenes were detected (**figure 24B and D**). The extracellular content in viniferin was slightly higher in the triple combination treatment when compared to MeJa and beta-MCD only.

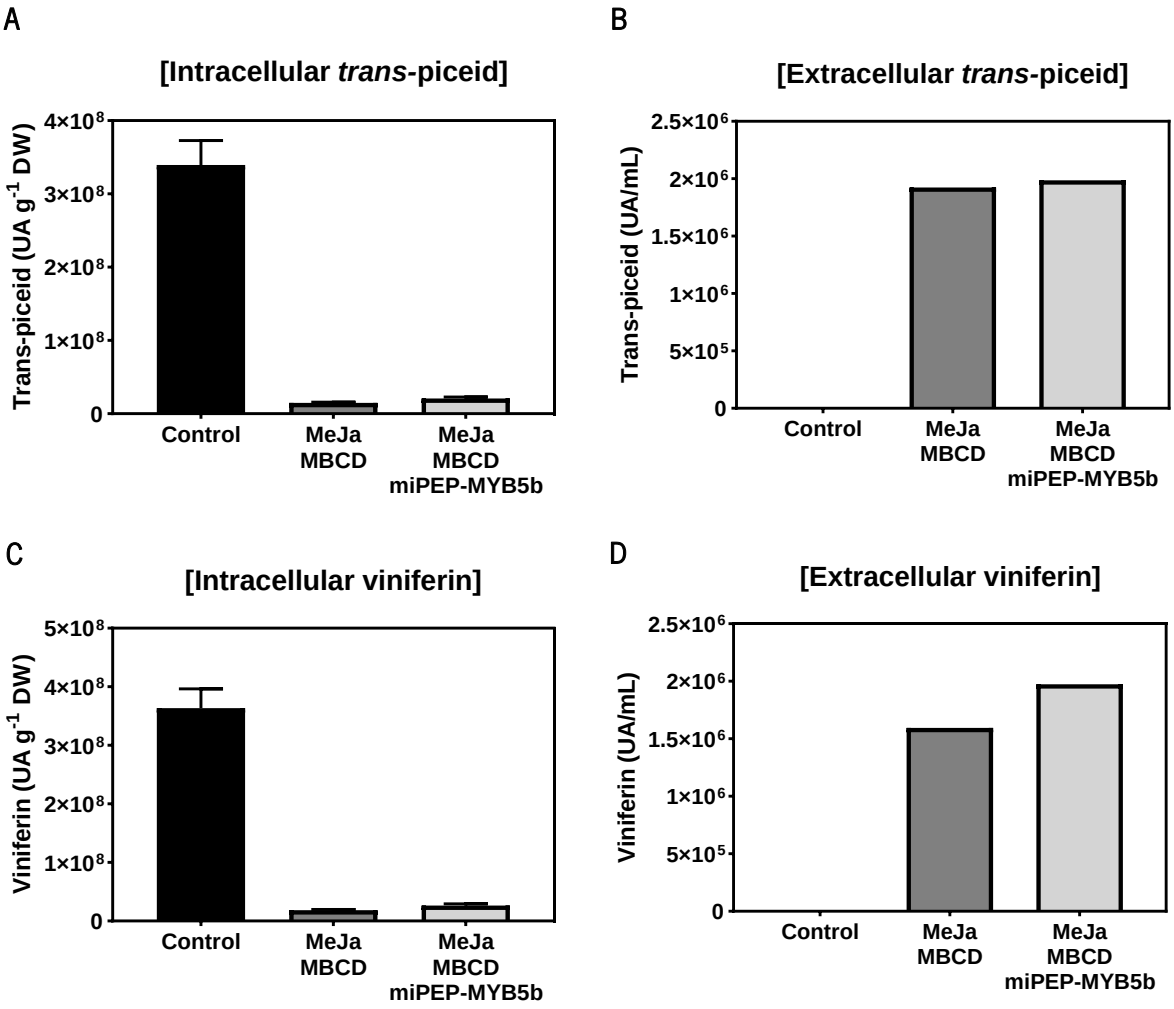

Figure 24. Effect of the exogenous application of Methyl Jasmonate, Methyl-β-cyclodextrin and miPEP-MYB5b (0.1 µM) on intracellular *trans*-piceid (A), extracellular *trans*-piceid (B), intracellular viniferin (C) and extracellular viniferin (D) content after 3 d of treatment in suspension-cultured grape berry cells. *Trans*-piceid and viniferin content is represented as arbitrary units per g of dry weight (DW).

3.6.1. Transcriptional changes in Gamay cells induced by the exogenous application of Methyl Jasmonate, beta-methylcyclodextrin and miPEP-MYB5b

3.6.1.1. Transcriptional changes in flavonol synthesis

Exogenous application of the treatment with MeJa and beta-MCD lead to a 40% decrease of *VvFLS1* expression levels, when compared to treatment with MeJa and beta-MCD only (**figure 25**). It is noteworthy that in that condition, the expression of *VvFLS1* is about 75-fold higher than in control cells.

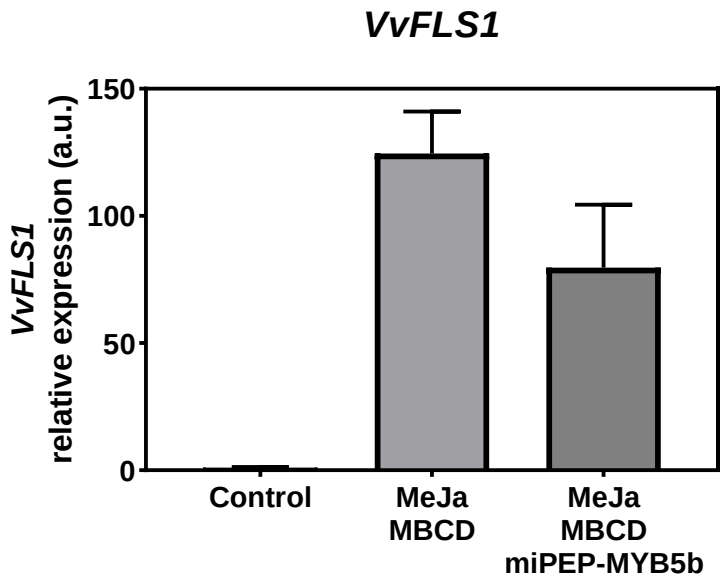

Figure 25. Effect of the exogenous application of Methyl Jasmonate, Methyl-β-cyclodextrin and miPEP-MYB5b (0.1 μM) in the transcript levels of *VvFLS1* after 3 d of treatment in suspension-cultured grape berry cells and without treatment (Control). Gene expression analysis, by real-time qPCR was normalized with the expression of reference gene *VvACT1* and *VvGAPDH*. Values are the mean ± SEM.

3.6.1.2. Transcriptional changes in proanthocyanidin and flavan-3-ol synthesis

The triple combination led to a 65% decrease in the expression levels of *VvLAR2* when compared to the treatment with double combination (**figure 26A**). Also, in both conditions the expression of *VvLAR2* was very significantly upregulated in comparison to the control. Likewise, the expression of *VvANR* was significantly higher than that in the control and approximately 35% lower in cells subjected to the triple combination, when compared to the treatment with double combination (**figure 26B**).

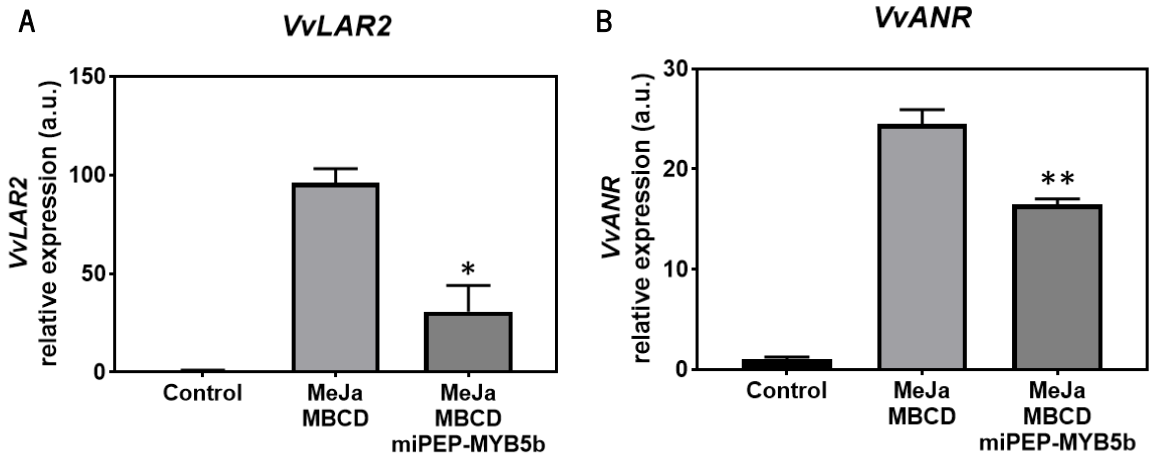

Figure 26. Effect of the exogenous application of Methyl Jasmonate, Methyl-β-cyclodextrin and miPEP-MYB5b (0.1 μM) in the transcript levels of *VvLAR2* (A) and *VvANR* (B) after 3 d of treatment in suspension-cultured grape berry cells and without treatment (Control). Gene expression analysis, by real-time qPCR was normalized with the expression of reference gene *VvACT1* and *VvGAPDH*. Values are the mean ± SEM. Asterisks indicate statistical significance (Student's t-test; * $P < 0.05$, ** $P < 0.01$ between the triple and double combination of elicitors).

3.6.1.3. Transcriptional changes in anthocyanin synthesis

Transcriptional analysis of *VvUFGT1* in cells treated with triple combination showed a 50% decrease when compared to the treatment with the double combination, but highly increased comparing to control cells (**figure27A**). The expression levels of anthocyanin vacuolar transporter *VvMATE1* in cells subjected to the triple combination were slightly but not statistically significantly increased comparing to those of cells with the double treatment, while the expression of *VvABCC1* was slightly decreased (**figure 27B and C**). Finally, the expression of the anthocyanin degradation-involved peroxidase *VvPRX31* in cells treated with triple combination displayed a slight non-statistically significant decrease, when compared to treatment with double combination (**figure 27D**).

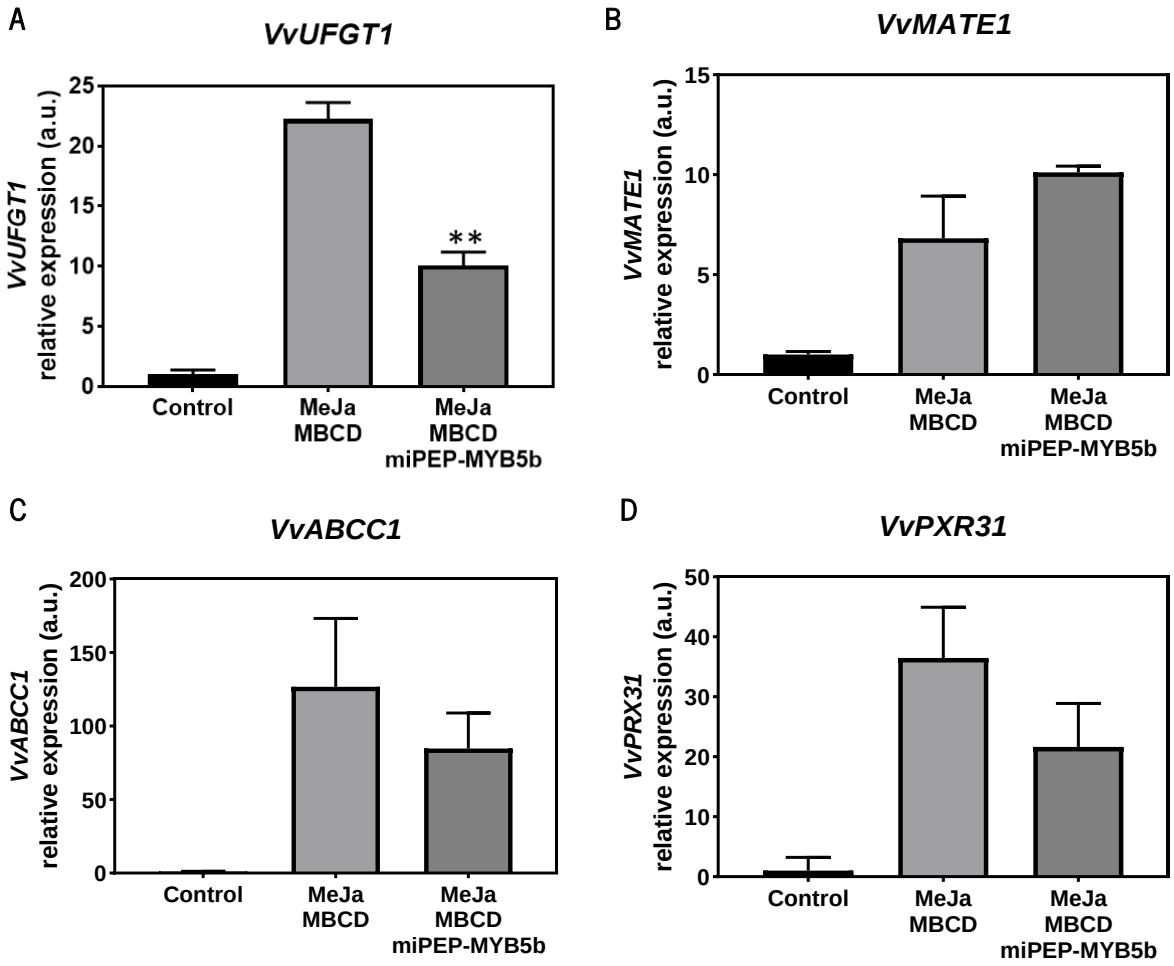

Figure 27. Effect of the exogenous application of Methyl Jasmonate, Methyl-β-cyclodextrin and miPEP-MYB5b (0.1 μM) in the transcript levels of *VvUFGT1* (A), *VvMATE1* (B), *VvABCC1* (C) and *VvPRX31* (D) after 3 d of treatment in suspension-cultured grape berry cells and without treatment (Control). Gene expression analysis, by real-time qPCR was normalized with the expression of reference gene *VvACT1* and *VvGAPDH*. Values are the mean ± SEM.

3.6.1.4. Transcriptional changes in stilbene pathway

In the stilbene pathway, transcript abundance of *VvSTS1* in cells treated with the triple combination was slightly but non-statistically significant higher to that of cells treated with the double combination (figure 28).

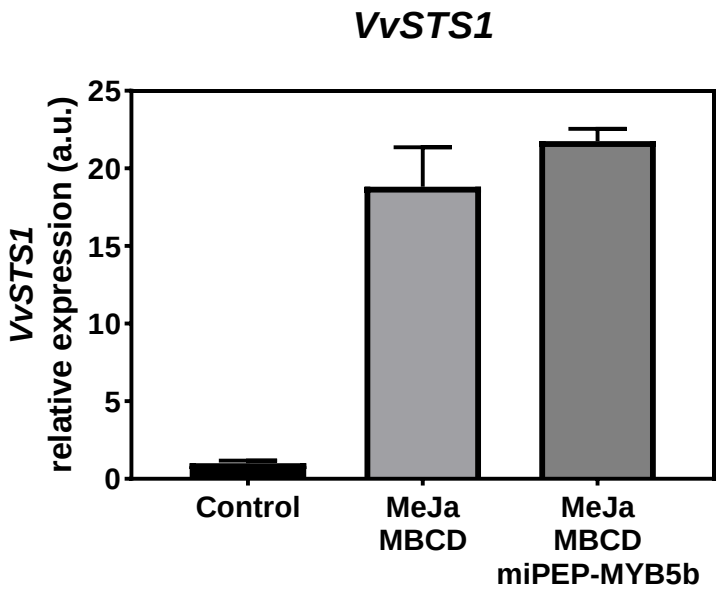

Figure 28. Effect of the exogenous application of Methyl Jasmonate, Methyl-β-cyclodextrin and miPEP-MYB5b (0.1 μM) in the transcript levels of *VvSTS1* after 3 d of treatment in suspension-cultured grape berry cells and without treatment (Control). Gene expression analysis, by real-time qPCR was normalized with the expression of reference gene *VvACT1* and *VvGAPDH*. Values are the mean ± SEM.

4. DISCUSSION

4. Discussion

V. vinifera has an array of phenylpropanoid compounds, including polyphenols (flavonoids and stilbenes), that assume multiple roles during plants life cycle. Grape berry secondary metabolites present key roles in plant defense responses and with numerous health benefits in human life. Stilbenes are classified as phytoalexins because of its capacity to restrain fungal growth. Grapevine stilbenes synthesis is induced by abiotic stress factors and these compounds hold valuable effects in preventing heart diseases and cancer due to their powerful antioxidant activities (Tavares et al., 2013). Several factors, including environmental conditions, affect the total grape berry polyphenolic composition. In the past few years, elevated temperatures are events with increased occurrence. Thus, overall berry quality and, ultimately, many industries that depend on this crop quality, are threatened (Jones and Storchmann, 2005).

As mentioned in the introduction section, plant miRNAs regulate the expression of numerous genes either by repression of protein production (reversible manner) or transcript cleavage (irreversible manner) (Voinnet, 2009). Recently, Lauressergues and his group (Lauressergues et al., 2015) discovered that pri-miRNAs, initially assumed as "junk DNA", hold the capacity to encode small regulatory peptides (miPEPs). Even though the mechanisms where pri-miRNA undergo either processing into miRNA or into miPEPs are still not fully understood, this discovery exposes an unexpected function of pri-miRNAs sequences and reveals another layer of gene regulation, without the need for genetic transformation. The recent study of Lauressergues and colleagues also revealed that the overexpression of miPEP171b in *Medicago truncatula* stimulated the accumulation of endogenous miR171b, resulting in differences in root development. Furthermore, in soybean (*Glycine max*), exogenous application of specific synthetic miPEPs demonstrated positive impact in nodule formation. Remarkably, plant treatments with synthetic micropeptides can strongly affect plant phenotype due to this positive regulation. This fact brings to light a possible strategy of gene modulation with exogenous treatments, either in suspension-cultured cells or in *planta,* and an easy implementation on agronomical plants (Couzigou et al., 2016).

4.1. Exogenous addition of miPEP396a induced the accumulation of miR396a and consequent miR396a-mediated flavonoid synthesis downregulation and stilbene synthesis upregulation

Exogenous application of miPEP-MYB5b resulted in a decrease in the concentration of flavonoids, including anthocyanins, in grape berry cells, confirming that some kind of downregulation of flavonoid biosynthetic genes is occurring due to stimulation of the post-transcriptional silencing of *VvMYB5b*, possibly through inhibition by cleavage, by the action of the mature miR396a. Indeed, the application of miPEP-MYB5b was confirmed to lead to significantly higher abundance of miR396a, the mature version of the non-mature miRNA that putatively encodes for miPEP396a, frequently designated in this study as miPEP-MYB5b, and to a significant decrease of *VvMYB5b* expression. This validates the hypothesis that the exogenous application of miPEP396a (miPEP-MYB5b) could lead to the accumulation of pri-miR396a and, consequently, to increase its translation into miPEP396a and trigger the negative regulation of *VvMYB5b*. Thus, a positive loop of miPEP396a-miR396a is established resulting in a VvMYB5b action partly silenced.

The flavonoid pathway competes directly with the stilbene pathway. The flavonoid biosynthetic genes *VvLAR1, VvLAR2, VvANR, VvFLS1, VvCHS1* and *VvCHI* were downregulated by miPEP-MYB5b treatment at either the concentration of 0.1 or 0.5 µM. The work by Deluc et al. (2008) concluded that VvMYB5b is able to activate the promoters of *VvLAR1*, *VvLDOX*, *VvCHI*, *VvANR* and *VvF3'5'H*. Chalcone synthase (CHS) in one side and stilbene synthase (STS) on the other side use the same substrate deriving from the phenylpropanoid pathway, alternatively directing the production of flavonoids or stilbenes, respectively. Thus, that downregulation of many molecular mechanisms involved in the flavonoid synthesis pathway contributed to the decrease of total flavonoid content in miPEP-treated cells. However, *VvLDOX* and *VvF3'5'H* genes did not respond to miPEP396a as we expected. This could be due to compensation mechanisms to assure a certain amount of compounds inside the cell and to maintain secondary metabolism in normal conditions, as other transcription factors also drive the regulation of these pathways. In fact, other transcription factors, including VvMYB5a, also modulate *VvLDOX* expression. Thus, silencing one regulator may be occasionally overcome by endogenous activation of another.

Concordantly, stilbene intracellular content increased with both concentrations of miPEP but 0.1 µM miPEP led to a higher content of a viniferin quantified by HPLC. Furthermore, *VvSTS1* expression increased effectively with 0.1 µM miPEP-MYB5b but had no significant changes with 0.5 µM concentration of miPEP. Thus, 0.1 µM concentration of miPEP is more effective in stimulating this

particular mechanism of the stilbene pathway. Still, stilbene synthesis increase at the expense of flavonoids decrease as well as the frequent repression of molecular mechanisms involved in flavonoid synthesis were overwhelmingly clear in cells treated with either 0.1 µM or 0.5 µM miPEP-MYB5b, as demonstrated in **tables 3** and **4**. Importantly, the expression of the peroxidase *VvPRX31*, putatively involved in anthocyanin degradation, decreased with 0.1 µM miPEP-MYB5b, so the lower anthocyanin concentration of miPEP-treated cells were not a result from any possible higher rate of anthocyanin degradation.

In general, these results confirm that miPEP396a exogenous application is certainly enhancing miR396a transcription, which leads to a more accentuated silencing of *VvMYB5b* and, ultimately, of VvMYB5b-activated genes. This silencing is indirectly leading to stilbene pathway upregulation, at least in part as *VvSTS1* expression levels increased, which is corroborated by the higher stilbene content in cells treated with miPEP396a.

Table 3. Compilation of the changes induced by the exogenous application of miPEP-MYB5b in the concentration of secondary metabolites.

Concentration of secondary metabolites		**0.1 µM miPEP-MYB5b**	**0.5 µM miPEP-MYB5b**
[Anthocyanin]$_{total}$		↓	↓
[Anthocyanin] (HPLC)	Anthocyanin 1	-	↑
	Anthocyanin 2	-	↑
	Anthocyanin 3	↓	↓
[Stilbenes] (HPLC)	*Trans*-piceid	-	-
	Viniferin	↑	↑
[Phenolics]$_{total}$		↓	↓
[Flavonoids]$_{total}$		↓	↓
Quercetin-3-glucoside (HPLC)		↓	↓
Epicatechin (HPLC)		↓	↓
Narigenin (HPLC)		↓	↓

Table 4. Compilation of the changes induced by the exogenous application of miPEP-MYB5b in the expression levels of key genes involved in the phenylpropanoid, stilbene, flavonoid, anthocyanin and norisoprenoid pathways.

Pathway	Branch	Gene	0.1 µM miPEP-MYB5b	0.5 µM miPEP-MYB5b
Phenylpropanoids		VvPAL1	↑	↑
		VvC4H	↑	-
		VvDAHPS1	↑	↑
Stilbenes		VvSTS1	↑	-
Flavonoids	Flavan-3-ols/ Proanthocyanidins synthesis	VvLAR1	↓	↓
		VvLAR2	↓	↓
		VvANR	-	↓
	Flavonols	VvFLS1	-	↓
		VvCHS1	-	↓
		VvCHS3	-	↑
		VvCHI	↓	↓
		VvF3'5'H	↑	↑
	General	VvDFR	↓	↓
		VvLDOX	↑	↑
	Anthocyanins	VvUFGT1	-	↓
Anthocyanin transport	Stabilization	VvGST4	↑	↓
	Transport	VvABCC1	↑	↑
		VvMATE1	↓	↓
	Degradation	VvPRX31	↓	-
Norisoprenoids		VvCCD4	-	↑

4.2. Could miPEP-MYB5b accentuate stilbene overproduction with MeJa and beta-MCD?

The miPEP-MYB5b application in combination with secondary metabolism elicitors MeJa and beta-MCD led to a slight increase of extracellular stilbene content (trans-piceid and viniferin) **(table 5)**, when compared to treatment with only MeJa and beta-MCD. *VvSTS1* was also stimulated when compared to treatment with double combination (**table 6**). Moreover, *VvLAR2*, *VvANR*, *VvUFGT1* and *VvPRX31* were downregulated. However, the increment in stilbene synthesis was significantly inferior to that induced by the addition of MeJa and beta-MCD. These preliminary results suggest that miPEP-MYB5b could possibly be used with other strategies to stimulate the stilbene synthesis *in vitro*. However, this approach could still be optimized in order to make good use of this strategy and possibly advance into an *in planta* treatment as well.

Table 5. Compilation of the changes induced by the exogenous application of triple treatment with MeJa, BMCD and miPEP-MYB5b in the concentration of secondary metabolites, when compared to double treatment.

Concentration of secondary metabolites		MeJa+MBCD+ miPEP-MYB5b (Intracellular)	MeJa+MBCD+ miPEP-MYB5b (extracellular)
$[Anthocyanin]_{total}$		↑	↑
[stilbenes]	*Trans*-piceid	-	↑
	Viniferin	-	↑
$[Phenolics]_{total}$		↑	-
$[Flavonoids]_{total}$		↑	↑

Table 6. Compilation of the changes induced by the exogenous application of triple treatment in the expression levels of key genes involved in the phenylpropanoid, stilbene, flavonoid, anthocyanin and norisoprenoid pathways, when compared to double treatment.

Pathway	Branch	Gene	MeJa+MBCD+ miPEP-MYB5b
Phenylpropanoids		VvPAL1	↓
		VvC4H	↑
		VvDAHPS1	↑
Stilbenes		VvSTS1	↑
Flavonoids	Flavan-3-ols/ Proanthocyanidins synthesis	VvLAR1	↑
		VvLAR2	↓
		VvANR	↓
	Flavonols	VvFLS1	↓
		VvCHS1	↑
		VvCHS3	↑
	General	VvDFR	↑
		VvLDOX	↓
	Anthocyanins	VvUFGT1	↓
Anthocyanin transport	Stabilization	VvGST4	-
	Transport	VvABCC1	↓
		VvMATE1	↑
	Degradation	VvPRX31	↓
Norisoprenoids		VvCCD4	↓

5. Conclusions and future perspectives

In this study, the exogenous application of miPEP-MYB5b (miR396a) provoked a downregulation of flavonoid biosynthesis. We tested two concentrations of miPEP-MYB5b and analyzed which concentration would induce the stilbene pathway more effectively. Recurring to a combination of molecular and biochemical approaches, results showed that 0.1 µM miPEP-MYB5b was more effective in enhancing stilbene biosynthesis as this concentration was the only one that increased *VvSTS1* expression levels, highly decreased *VvMYB5b* expression levels and increased viniferin intracellular concentration, despite a stilbene synthesis stimulating and flavonoid inhibiting effect also observed with the application of 0.5 µM miPEP-MYB5b. This upregulation of stilbene pathway seems to be an indirect effect of miPEP-MYB5b putatively inhibiting transcription factor MYB5b, an activator of promoters of many structural genes in the flavonoid pathway (a pathway that directly competes with stilbene pathway). Therefore, miPEP-MYB5b triggered higher stilbene content and lower concentration of flavonoids due to miR396a-mediated negative regulation of *VvMYB5b* and the respective genes activated by this transcription factor in the flavonoid pathway.

Taking this together, a miPEP-based treatment appears to be a promising new strategy to modulate plant secondary metabolism and improve plant cell characteristics by enhancing the synthesis of certain compounds, without the need for genetic engineering. Applied *in planta*, it could hypothetically improve berry quality or resistance of grapevine to biotic stress because stilbenes exhibit biological roles in the response to pathogen attacks as miPEP-MYB5b led to higher stilbene concentration *in vitro* (Viret et al., 2018*,* Chang et al., 2011).

Still, further studies are required to understand miPEP-MYB5b action in post-transcriptional silencing, such as a thorough metabolomic analysis, quantification of other secondary metabolites by HPLC and enzymatic activity analysis of enzymes which genes are regulated by MYB5b. In addition, the general micropeptide-mediated post-transcriptional silencing information is scarce. Little is known about how micropeptides are transported across the cell wall and cell membranes into cytoplasm or nucleus or how they are stabilized inside the cell. Besides, it would be interesting to study which non-mature miRNAs encode miPEPs and what effects they all have in plants. In the recent years, there have been more records of climate changes that threaten not only grapevine cultivation but all plant crops. Further studies could lead to potential micropeptides to improve all kinds of crops quality. On the other hand, the recent trend on natural products requires investigations into plant extraction of these compounds and into sustainable forms of producing them. As miPEP-MYB5b stimulates stilbene production, more

studies could lead to the creation of a sustainable strategy to enhance biosynthesis of stilbenes as nutraceutical compounds for industrial application.

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