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Identification and characterization of genes involved in stilbene biosynthesis and modification in *Vitis vinifera*

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Table of contents

Summary

Stilbenes are plant secondary metabolites from the polyphenols group found in 72 unrelated plant species. Among those plants is *Vitis vinifera*, an important crop plant in wine regions all across the world. The natural functions of stilbenes include the reaction to stress factors like UV-radiation and mechanical injury or defense reactions against bacterial, fungal or other pathogens. One of the most problematic pathogens in the wine regions in Germany is *Plasmopara viticola*, causing downy mildew, which leads to great crop losses. Aside from that, stilbenes have gotten attention in the recent years for their potent health beneficial properties. A multitude of studies, mainly pre-clinical trials, have indicated that stilbenes can delay aging parameters, help in the prevention of diseases including cancer or diabetes and are effective against cardiovascular diseases.

While much attention is given to the functions of stilbenes, both *in planta* and in the medical or nutritional supplement areas, and the structures and occurrences of stilbenes are mostly known, the biosynthetic pathway leading to this wide variety of modified stilbenes remains largely unknown. The precursors of the stilbenes derive from the phenylpropanoid pathway, where the stilbene synthase, as first dedicated enzyme of the pathway produces *trans*-resveratrol. Apart from that, only one other enzyme, a resveratrol-O-methyl transferase and the two transcription factors (TFs) VvMYB14 and VvMYB15 are known and characterized. Other enzymes, for example for glycosylation, polymerization or other modification reactions remain yet to be found, as do dedicated transport proteins.

Different *V. vinifera* tissues overexpressing VvMYB15, one of the two TFs that were shown to be important regulators of the stilbene biosynthesis pathway, were used to run two microarrays prior to this project. From this database, candidate genes with potentially important predicted functions were identified by their upregulation. The selected genes were then filtered by the means of *in silico* analysis and promoter induction assays before the correlation of their gene expression with the content of modified stilbenes, expected to be produced by their reaction, was investigated. This was done in *V. vinifera* berries of different developmental stages as well as leaf discs infected with *P. viticola* and allowed for the closer connection of several candidate genes with their expected products, while also giving valuable insight into the potential role of the stilbenes, and thus the genes, during development or in a defense against infection. In the last part of this thesis, the most promising candidate genes, three glycosyltransferases and a laccase, underwent closer biochemical characterization by expression in bacteria, yeast or tobacco and subsequent enzymatic assay or direct HPLC or UHPLC/MS analysis. While the full characterization of the candidate genes remains to be completed, promising results for the glycosyltransferases were obtained concerning the metabolization of resveratrol and other substrates and for the involvement of the laccase and viniferin in defense reactions.

Zusammenfassung

Stilbene sind pflanzliche Sekundärmetabolite aus der Gruppe der Polyphenole, die in 72 nicht verwandten Pflanzenarten vorkommen. Zu diesen Pflanzen gehört auch *Vitis vinifera*, eine wichtige Kulturpflanze in Weinregionen auf der ganzen Welt. Zu den natürlichen Funktionen der Stilbene gehören die Reaktion auf Stressfaktoren wie UV-Strahlung und mechanische Verletzungen oder Abwehrreaktionen gegen bakterielle, pilzliche oder andere Krankheitserreger. Einer der problematischsten Erreger in den deutschen Weinregionen ist *Plasmopara viticola*, der Falsche Mehltau, der zu großen Ernteausfällen führt. Abgesehen davon haben Stilbene in den letzten Jahren wegen ihrer potenten gesundheitsfördernden Eigenschaften Aufmerksamkeit erregt. Eine Vielzahl von Studien, vor allem präklinische Versuche, haben darauf hingewiesen, dass Stilbene Alterungsparameter verzögern können, bei der Vorbeugung von Krankheiten wie Krebs oder Diabetes helfen und gegen Herz-Kreislauf-Erkrankungen wirksam sind.

Während den Funktionen der Stilbene sowohl *in planta* als auch im Bereich der Medizin oder der Nahrungsergänzung viel Aufmerksamkeit geschenkt wird und die Strukturen und Vorkommen der Stilbene weitgehend bekannt sind, ist der Biosyntheseweg, der zu dieser großen Vielfalt an modifizierten Stilbenen führt, noch weitgehend unbekannt. Die Vorstufen der Stilbene entstammen dem Phenylpropanoidsyntheseweg, wobei die Stilbensynthase als erstes dediziertes Enzym des Weges *trans*-Resveratrol produziert. Daneben sind nur ein weiteres Enzym, eine Resveratrol-O-Methyl-Transferase und die beiden Transkriptionsfaktoren (TFs) VvMYB14 und VvMYB15 bekannt und charakterisiert. Weitere Enzyme, z. B. für die Glykosylierung, Polymerisation oder andere Modifikationsreaktionen, sind noch nicht bekannt, ebenso wenig wie spezielle Transportproteine.

Verschiedene *V. vinifera*-Gewebe, die VvMYB15, einen der beiden TFs, die sich als wichtige Regulatoren des Stilben-Biosynthesewegs erwiesen haben, überexprimieren, wurden im Vorfeld dieses Projekts für die Durchführung zweier Microarrays verwendet.

Aus dieser Datenbank wurden Kandidatengene mit potentiell wichtigen vorhergesagten Funktionen durch ihre Hochregulierung identifiziert. Die ausgewählten Gene wurden dann mit Hilfe von *in silico*-Analysen und Promotor-Induktions-Assays gefiltert, bevor die Korrelation ihrer Genexpression mit dem Gehalt an modifizierten Stilbenen, von denen erwartet wird, dass sie durch ihre Reaktion produziert werden, untersucht wurde. Dies geschah in *V. vinifera*-Beeren verschiedener Entwicklungsstadien sowie in mit *P. viticola* infizierten Blattscheiben und ermöglichte die nähere Verknüpfung mehrerer Kandidatengene mit ihren erwarteten Produkten, wobei auch wertvolle Einblicke in die mögliche Rolle der Stilbene und damit der Gene während der Entwicklung oder bei der Abwehr einer Infektion gewonnen werden konnten. Im letzten Teil dieser Arbeit wurden die

vielversprechendsten Kandidatengene, drei Glykosyltransferasen und eine Laccase, einer näheren biochemischen Charakterisierung durch Expression in Bakterien, Hefe oder Tabak und anschließendem enzymatischen Assay oder direkter HPLC- oder UHPLC/MS-Analyse unterzogen. Während die vollständige Charakterisierung der Kandidatengene noch aussteht, wurden für die Glykosyltransferasen vielversprechende Ergebnisse hinsichtlich der Metabolisierung von Resveratrol und anderen Substraten sowie für die Beteiligung der Laccase und des Viniferins an Abwehrreaktionen erzielt.

1 Introduction

1.1 *Vitis vinifera* – a historical crop with modern challenges

Vitis vinifera, the common grapevine, is a plant species native to the Mediterranean region, central Europe and central Asia, belonging to the *Vitis* genus, which itself contains 79 species and can be found on the European, Asian, African and American continents, mainly in the northern hemisphere (Vivier, 2000). The genus *Vitis* as such is divided into two subgenera "Euvitis" and "Muscadinia". While the latter only contains three species, the Euvitis subgenus includes the most well-known and important *Vitis* species, including *Vitis vinifera* (Mullins et al., 1992). *Vitis vinifera* can be divided into the two subspecies *V. vinifera sylvestris*, the so called "wild" subspecies and *V. vinifera sativa*, which is representative of the domestication history of grapevine (Terral et al., 2010).

Humanity has been using the grapevine berries for a variety of purposes for thousands of years. It is thought that domestication occurred east of the Mediterranean area and spread from there to southern and central Europe via Egypt and Greece, along early trade and colonization routes. The uses of the plant include direct consumption of the berries, or even leaves in some cultures, as well as processing of the berries into more durable sweets. The most famous use however is wine making which is said to have occurred already since pre-historic times (McGovern et al., 1996; Valamoti et al., 2007; Terral et al., 2010).

Presently, grapevine is one of the most important agricultural fruit crops, with over 7 million hectares used for grape production worldwide and approximately 27 billion liters of wine produced in 2014 alone. While for both numbers there were no large changes during the last three decades, the production area and market share began to shift from traditional wine growing countries like Spain, France and Italy towards newcomers in the field, most notably China. Europe still holds about 54 % of the world's vineyards, but this is a decrease from the over 60 % it accounted for just a decade earlier (Food and Agriculture Organization of the United Nations, 2020; International Organisation of Vine and Wine, 2020).

Due to its economic importance and rising scientific interest, the grape genome was among the first genomes of economically important plants to be fully sequenced, and was in fact the first flowering plant to reach that state, which it did in 2007. A 'Pinot Noir' line, bred to high homozygosity, (PN40024) was used for the sequencing and it was revealed that on the 19 chromosome pairs (2n=38) of *V.vinifera* there were an estimated 26,000 genes present in the 487 megabases of the genome (Jaillon et al., 2007; Grape Genome Browser, 2020).

This opened new opportunities in grapevine research on a genetic level and increased the pace with which solutions for the many diseases and other vulnerabilities of *V. vinifera* could be found.

The history of research on grape diseases reaches back to the mid-19th century, when the exchange of European and American *Vitis* species began, due to the increased development in the overseas colonies. Winegrowers on the new continent realized that old-world varieties were not suitable to be grown in America, since they succumbed to diseases or adverse growth conditions and after the introduction of new-world diseases to France, and then all of Europe, those same diseases lead to immense losses in European wine production. Whereas scientists managed to breed resistant rootstocks against the insect *Daktulosphaira vitifoliae* which causes grape phylloxera, two other diseases, downy mildew and powdery mildew, have been haunting winegrowers for over 150 years and are usually kept at bay by the application of fungicides. The fact that American *Vitis* species showing some resistance against *Erysiphe necator* (the fungus causing powdery mildew) and *Plasmopara viticola* (the oomycete causing downy mildew) cannot be as easily exploited by breeders as in the case of phylloxera has thus given rise to the field of grape resistance research (Gessler et al., 2011; Töpfer et al., 2011; Benheim et al., 2012).

Since the *Vitis* species that show resistance to diseases usually have properties that cause the wine made from their grapes to be of low quality, it has been attempted to cross the high-quality European *V. vinifera* varieties with American, or lately also with Asian Vitis species in order to produce progeny that combine the traits of good tasting wine and high resistance to diseases in the field. These hybrids were produced from about the 19th to the first half of the 20th century, mainly privately in France and in research centers in Germany. These early hybrids rarely yielded wine of high quality, were not commercially successful, and thus mostly vanished from the vineyards of Europe in the second half of the 20th century (Paul, 2002; Töpfer et al., 2011). The cause of these difficulties is the circumstance that good wine quality is a combination of many factors and thus much more complicated to select for and breed than resistances alone. In order to retain the sequences of the genome that are responsible for the resistances but at the same time eliminate as much as possible low-quality properties of the "wild" or "American/Asian" genome in order to preserve the good quality traits for wine quality, many back crossings with European parents are needed. This is achieved with about six generations, which, due to the plants needing three years to produce their first grapes combined with selection in each generation, takes about 30 years for the development of a new variety. The selection for quality is traditionally done by production of small test quantities of wine, the so-called micro-vinification, and subsequent testing by expert panels, which also adds to the long-time requirement (Bouquet et al., 2000; Töpfer et al., 2011; Montaigne et al., 2016).

With the rise of molecular marker techniques in the 1990s, it became realistic to expect a shortening of this time requirement through their use in breeding efforts. At first, it was only possible to distinguish specimen by restriction fragment length polymorphism (RFLP) or randomly amplified polymorphic DNA (RAPD). The use of simple sequence repeats (SSRs) as molecular markers made it

possible to search for such markers that accompany a desirable trait, like resistance or even single aspects of good wine quality, although the interplay of all the known and yet unknown factors to good quality still make this a complex topic (Beckmann and Soller, 1990; Thomas and Scott, 1993). In recent years, researchers have published various loci for resistances and other traits, respectively markers for those sites, including resistance to *P. viticola* (Welter et al., 2007; Di Gaspero et al., 2012), *E. necator* (Barker et al., 2005), monoterpene content (Battilana et al., 2009) or even berry size (Doligez et al., 2002).

It has already occurred however that resistant plants in the field have been infected with strains, e.g. of *P. viticola*, that were able to overcome one resistance trait, indicating that it will be important for the long-term durability of grapevine cultivars in vineyards to have a combination of resistance loci (Eibach et al., 2007). This has already been achieved for some first cultivars, e.g. 'Sauvignac' and 'Calardis Blanc' (Vitis International Variety Catalogue VIVC, 2020). While a combination of resistance loci in one plant greatly increases its degree of resistance against pests, a much-reduced amount of pesticide application might still be in order to greatly disrupt the development of counter resistances by the pathogens (Eisenmann et al., 2019).

This combination of knowledge of the genome and molecular techniques not only benefits targeted breeding efforts, but of course also allows for searching, mapping, comparison and thus targeted cloning and finally identification of genes that are of interest for other research fields within *V. vinifera*.

1.2 Plant secondary metabolism and polyphenols – a versatile toolkit for the sessile life forms

Plants are unable to change their location in the case of unfavorable conditions and therefore need to be more adaptable than animals. This is achieved in many ways, one of them being secondary metabolites they produce, which include over 200,000 different molecular products. For the longest time they were considered by-products of the "primary" metabolite production, until early research in the field from the middle of the 20th century started to reveal their many different functions (Reznik, 1960; Barz and Köster, 1981; Hartmann, 1996). Those functions, for example defense from herbivores, rely on secondary metabolites, which themselves are produced from primary metabolites. This underlines the different nature of both metabolite classes: Primary metabolites are essential for growth and development of the plants, while secondary metabolites ensure survival and adaption to external influences (Reznik, 1960; Hartmann, 2007).

Due to the immense variety of secondary metabolites, there are many ways to classify them. One popular way is by biosynthetic pathway, which leads to three classes: the phenolics, the terpenes and steroids and thirdly the alkaloids (Harborne, 1999; Bourgaud et al., 2001).

Secondary metabolites as defense compounds often are subject to co-evolution with herbivores or pathogens. Therefore, it is not unusual that some herbivores or pathogens become immune to the defense compound, find a way around, or counter it in another way (Wink, 1988; Cornell and Hawkins, 2003; Theis and Lerdau, 2003).

In general, there are three defense strategies that plants utilize. The first option is the constitutive expression of defense compounds, although this of course is quite resource-heavy and potentially damaging for the plant cells themselves. The second method is to provide precursor substances and enzymes in separate compartments, which then act together when combined in specific defense situations. This still requires the resources, but eliminates the damage threat. Lastly, production of the secondary metabolites involved in the defense reaction can be induced situation-specifically. This is the most economical, but of course also slowest strategy (Hartmann, 2004; Hartmann, 2007; Isah, 2019).

The enzymes of the biosynthetic pathways that are responsible for the synthesis and modification of certain secondary metabolite classes must have evolved from some precursors, since they are fulfilling their functions in plants, or even only in certain plants. Two possibilities here are the evolution from enzymes of the primary metabolism or of other, older, secondary metabolism pathways. Indeed there are examples of pathways overlapping, like the cholesterol and phytosterol pathways (Sonawane, 2016) or still having primary functions, although being primarily known for the secondary metabolism role, like the flavonoids in auxin transport (Buer and Muday, 2004). Also important in the development of metabolic pathways is gene duplication, where the copied gene is then not inactivated but rather evolves new functions and is implemented in new pathways (Kliebenstein et al., 2001; Benderoth et al., 2006; Ober, 2010). This final example was also the case with the development of the polyketide synthases, where, from a duplication of a chalcone synthase gene, the group of chalcone synthases as well as a group of related enzymes were formed, including the stilbene synthases that are important in this work (Tropf et al., 1994; Liu et al., 2007).

1.3 Stilbenes – important players in plant defense and upcoming healthy consumable for humans

1.3.1 Stilbene occurrence in plants

Stilbenes are secondary metabolites that derive from the phenylpropanoid pathway, which itself gives rise to many substances with various functions, including stress responses and defense reactions (Dixon and Paiva, 1995). Stilbenes are present only in 72 plant species across 12 non-related families throughout the plant kingdom (Sotheeswaran and Pasupathy, 1993; Pawlus et al., 2012). Similar to secondary metabolites in general, stilbenes comprise a large variety of molecules. Most stilbenes are derived from *trans*-resveratrol (3,5,4'trihydroxystilbene), which is the case for *V. vinifera*, although in other plants, such as from the *Pinus* or *Picea* genii, the structurally similar precursors pinosylvin or piceatannol are used in the stilbene biosynthetic and modification pathway (Morales et al., 2000).
Resveratrol was discovered in the mid-1900s in root extracts of Veratrum grandiflorum (Takaoka, 1940) and Polygonum cuspidatum (Nonomura et al., 1963), but has only experience increased scientific interest from the 1990s on (Baur and Sinclair, 2006).

1.3.2 Precursors of stilbene biosynthesis and related pathways

At the beginning of the synthesis of many secondary metabolites, indeed of many metabolites in plants in general, stands the shikimate pathway with its three products phenylalanine, tyrosine and tryptophan (Herrmann, 1995). The amino acid phenylalanine is both a product of the shikimate pathway and the substrate in phenylpropanoid pathway (Koukol and Conn, 1961; Herrmann, 1995).
The phenylpropanoid pathway then progresses from the produced amino acid phenylalanine to cinnamic acid through deamination by the enzyme phenylalanine ammonia lyase (Koukol and Conn, 1961) (Figure 1). After hydroxylation to p-coumarate by cinnamate 4-hydroxylase (C4H) (Schoch et al., 2001) and subsequent formation of p-coumaryl-CoA by the enzyme 4-coumarate:CoA ligase (4CL) (Lee et al., 1995; Ehlting et al., 1999), the pathway towards flavonoids and stilbenoids diverges from those towards other secondary metabolites, for example lignins. The other compound, beside p-coumaryl-CoA, that is needed to produce flavonoids and stilbenoids is malonyl-CoA, which is produced from acetyl-CoA by Acetyl-CoA carboxylase (ACC) (Wakil, 1958).

1.3.3 Stilbene synthesis, enzymes and structures

Both of the above-mentioned precursors are then used by the first committed enzyme for the stilbene biosynthesis pathway, which is called stilbene synthase (STS). This enzyme is a type III polyketide synthase and is closely related to the chalcone synthase (CHS) with which it forms this superfamily of enzymes (Schroder et al., 1988a; Austin et al., 2004). Chalcone synthases are found throughout the plant kingdom and usually produced by several different genes per plant. This allows them to fulfill a wide variety of functions and be expressed either in different tissues or upon different induction factors (Koes et al., 1989; Harborne and Williams, 2000). Since it was first reported that p-coumaroyl-CoA and malonyl-CoA are funneled into a specific pathway by an enzyme like CHS (Kreuzaler and Hahlbrock, 1972), much research into the specifics of those enzymes and reactions has been undertaken. Both CHS and STS first form a linear tetraketide intermediate from their precursor molecules (Figure 1). CHS then cyclizes the resulting molecule with an intramolecular Claisen condensation, leading to a structure with two carbon rings, of which the newly formed one is then subsequently aromatized in the next step. Thus, this enzymatic reaction results in a molecule with two benzole rings connected by three carbon atoms (C6-C3-C6). STS, which evolved from CHS, differs in that an intramolecular aldol condensation takes place one carbon atom further in, followed by a decarboxylative loss of the outermost carbon atom, resulting in the C6-C2-C6 structure of the *trans*-resveratrol molecule (Schroder et al., 1988a; Tropf et al., 1995; Austin et al., 2004). This synthesis from p-coumaroyl to *trans*-resveratrol is the most common variant, although some plants, like *Pinus sylvestris* show a slightly modified pathway. In this case, the STS enzymes seems to prefer cinnamoyl-CoA as a substrate and thus ends up forming the slightly different molecule pinosylvin (Table 1). If cinnamoyl is not available, it reverts to the original reaction, although less efficiently (Schanz et al., 1992; Schröder and Schröder, 1992). It has been shown that grapevine contains 48 stilbene synthases, which makes it the plant with the most STS genes and is in line with the multiple copies of many genes after gene duplications in grapevine (Falginella et al., 2010; Giannuzzi et al., 2011; Vannozzi et al., 2012). Furthermore, due to their similar nature and the identity of their substrates, it is not surprising, that CHS and STS compete for resources and seem to be regulated in opposing patterns in some cases (Vannozzi et al., 2012).

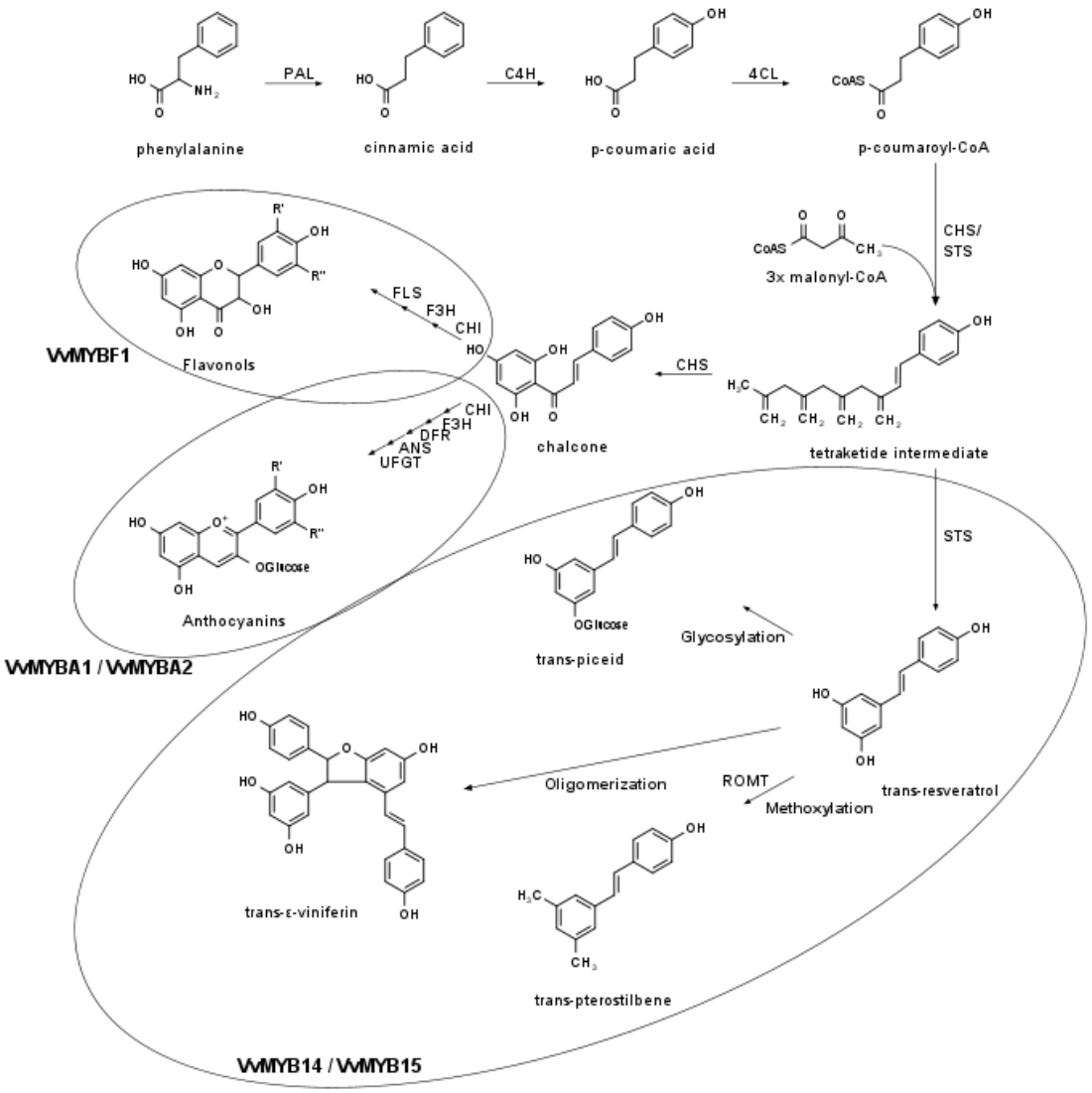


Figure 1: Simplified phenylpropanoid- and biosynthesis pathway of selected stilbenes and flavonoids in *Vitis vinifera* with corresponding VvMYB transcription factors.

Simplified depiction of the phenylpropanoid pathway starting at phenylalanine, which is derived from the shikimate pathway, and its branches from p-coumaroyl-CoA towards selected flavonoid and stilbene branches. Also depicted are the corresponding VvMYB transcription factors of the stilbene and flavonoid pathways. PAL: phenylalanine ammonia lyase, C4H: cinnamate 4-hydroxylase, CoA: coenzyme A, 4CL: 4-coumarate-CoA ligase, CHS: chalcone synthase, CHI: chalcone isomerase, F3H: flavonoid 3' hydroxylase, FLS: flavonol synthase, DFR: dihydroflavonol reductase, ANS: anthocyanidin synthase, UFGT: UDP-Glc:flavonoid-3-O-glucosyltransferase, STS: stilbene synthase and ROMT: resveratrol-O-methyltransferase. Figure according to information from Chong et al. (2009).

1.3.4 Regulation of the stilbene biosynthesis and involved transcription factors

This wealth of different enzymes involved in the biosynthesis of phenylpropanoids, especially flavonoids and stilbenes suggests that there should be a network in place to regulate their expression, for example with respect to developmental stage, organ or environmental influence. For flavonoids and stilbenes this mainly seems to happen at the transcriptional level with the help of transcription factors (TFs) (Weisshaar and Jenkins, 1998; Dubrovina and Kiselev, 2017). An important transcription factor family in plants and especially in grapevine are the MYB transcription factors, more precisely the R2R3 type sub-family (Matus et al., 2008). It has been previously shown that they regulate flavonoid as well as stilbene biosynthesis and modification (Stracke et al., 2007; Höll et al., 2013).

A gene of the MYB transcription factor type was first identified and cloned from avian myeloblastosis virus in 1982 (Klempnauer et al., 1982), whereas the first plant *MYB* gene was discovered 1987 in maize (Paz-Ares et al., 1987). In vertebrates there are three *MYB* genes (Weston, 1998), while the gene family has massively expanded in plants with the R2R3 type subfamily, to which *VvMYB14* and *VvMYB15* belong, being the most numerous in higher plants (Du et al., 2009). MYB TFs contain a conserved DNA binding domain, called the MYB domain. It is made up of between one and four imperfect amino acid sequence repeats (R), of which each is forming three α-helices. The second and third of these helices comprise a helix-turn-helix structure that has the function of intercalating with the major groove of the DNA strand (Ogata et al., 1996; Jia et al., 2004). The number and combination of the R-repeats are used to group the MYB TFs into different classes (Stracke et al., 2001). A prominent example of a group of secondary metabolites regulated by MYB TFs are the flavonoids. For some flavonoids however, they require co-factors, such as MYC proteins (basic helix–loop–helix (bHLH) domains) and conserved WD repeats (WDR) (Weisshaar and Jenkins, 1998; Stracke et al., 2001). Specific MYB TFs have been shown to regulate their distinct flavonoid classes, e.g. MYBA1 and MYBA2 being responsible for the anthocyanin production, MYBPA1 and MYBPA2 for proanthocyanidin and MYBF1 for flavonol (Figure 1). Additionally, there are also MYB TFs responsible for the unspecific parts of the flavonoid biosynthesis, namely MYB5a and MYB5b (Kobayashi et al., 2002; Downey et al., 2003; Bogs et al., 2007; Czemmel et al., 2009; Terrier et al., 2009). These regulations vary not only between tissues but also between developmental stages and other situations caused by external stimuli. Furthermore, it can be observed that differences in regulation between cultivars in *V. vinifera* cause different flavonoid levels, which in case of anthocyanins is obvious in color differences, but might, in case of other colorless substances have an impact on quality or taste (Walker et al., 2007; Matus et al., 2008). While the MYB TFs for the flavonoid biosynthesis pathways were already known, the transcription factors regulating stilbene biosynthesis and modification were only discovered in 2013. It was assumed that, due to the close relation of the pathways and the first specific enzymes, similar TFs would be responsible for the

regulation of STS and the potential downstream enzymes. Indeed, by investigating MYB TFs related to STS upregulation, two genes were found: *VvMYB14* and *VvMYB15*. Both showed co-expression with STS during infection with downy mildew, UV-C stress and in specific developmental stages. It was also shown that they activate the promoters of STS genes and thus the first transcription factors for the stilbene biosynthetic pathway were confirmed (Höll et al., 2013) (Figure 1). Similar observations have been made in other cultivars since then (Fang et al., 2014; Bai et al., 2019; Luo et al., 2019).

Another group of transcription factors that play a role in the stilbene metabolism, although so far less investigated, are the WRKY-TFs. They can be found in all higher plants and 59 WRKY TFs can be found in grapevine (Ülker and Somssich, 2004; Wang et al., 2014). They are defined by their DNA binding region, the WRKY domain with its zinc-finger motive, and are grouped into three main groups according to the number of WRKY domains present (de Pater, 1996; Rushton et al., 1996; Wang et al., 2014).

Similar to MYB TFs, they are associated with defense responses against pests and their respective signaling networks, as well as with responses to abiotic stress factors (Jiang and Deyholos, 2009; Rushton et al., 2010). Furthermore, they also show differences in spatial and temporal expression patterns during grapevine development (Wang et al., 2014). Four WRKY TFs in particular also showed either inducing function on STS promoters or even a combinatorial effect with VviMYB14, strongly suggesting that they, the MYB TFs and possibly other TF families are part of a regulatory network of stilbene metabolism (Vannozzi et al., 2018).

1.3.5 Modification reactions of resveratrol

Many secondary metabolites produced in plants subsequently undergo modification, changing their chemical properties and influencing, for example their function, transport or storage. This can include reactions like polymerization, glycosylation, acetylation, methylation, hydroxylation and many more, which all contribute to the creation of the enormous variety of plant secondary metabolites and their functions (Table 1).

One important form of modification is the glycosylation of secondary metabolites, catalyzed by enzymes from the glycosyltransferase family. Glycosyltransferases can be found throughout all life forms and contain thousands of known genes. The largest of these gene families, designated family 1, contains all the plant glycosyltransferases (Hughes and Hughes, 1994; Coutinho et al., 2003). Their functions *in planta* influence stability, solubility, subcellular localization, bioactivity and other properties. Those four examples tie into the general idea that the glycosylation can be used on active or even toxic compounds in order to control their potential to protect the plant and prepare it for situations where these compounds are for example needed in defense or stress responses. This might

be achieved by stabilizing reactive aglycones and even redirecting them into storage for later use (Jones and Vogt, 2001). Other functions include for example the modification of certain properties such as color. It was discovered for example that a glycosyltransferase is responsible for the typical blue color produced by an anthocyanin in gentian petals (Fukuchi-Mizutani et al., 2003). In citrus fruits on the other hand, glycosylation of limonoids can change the taste of the component from bitter to tasteless, showing the large influence of glycosylation reactions (Kita et al., 2000). Maybe not surprisingly, it has been shown that some glycosyltransferases can accept different substrates, while in other cases one compound can be modified by a number of glycosyltransferases. This might indicate that in vivo, two essential factors in these reactions are gene regulation and the availability of the substrate (Bowles et al., 2006). In *Vitis vinifera*, similar accumulations of glycosylated stilbenes have been shown in healthy tissues during ripening, upon UV-radiation as well as in defense against *Plasmopara viticola* (Waterhouse and Lamuela-Raventós, 1994; Adrian et al., 2000; Pezet et al., 2004b). This suggests that there are most likely several glycosyltransferases in *V. vinifera* that are involved in modification of stilbenes. There are three publications covering the investigation of glycosyltransferases which are able to process *trans*-resveratrol by glycosylation. One identified three glycosyltransferases from *V.vinifera*, cv. 'Macabeu' (Khater et al., 2012), while the two other groups found one of them also in Concord grape (*Vitis labrusca*) (Hall and De Luca, 2007) and in *Vitis amurensis* (Kiselev et al., 2017).

While these reactions cover the stored and thus more inactive forms of secondary metabolism and specifically stilbene modification, more active and task-specific forms are produced by methylation and polymerization.

Methylation of secondary metabolites in plants is done by methyltransferases, of which especially the O-methyltransferases are of importance, e.g. for lignin biosynthesis and compounds involved in stress reactions and defense (Inoue et al., 1998; Lam et al., 2007). As is the case with other enzymatic groups, methyltransferases show different levels of substrate specificity, with some for example specifically preferring certain flavonoids (Willits et al., 2004), while others process a wider variety, including phenylpropanoids, flavonoids and even stilbenes (Gauthier et al., 1998; Chiron et al., 2000a). A O-methyltransferase was discovered in *V. vinifera* that is involved in the production of pterostilbene, a methylated resveratrol which was already identified as important actor in defense against *P. viticola* and *Botrytis cinerea* in grapevine (Langcake et al., 1979; Langcake, 1981; Schmidlin et al., 2008).

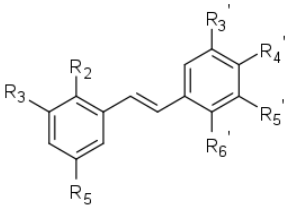
Similar to pterostilbene, viniferins were discovered and associated with plant defense and stress response. The researchers showed early that P. viticola infection as well as UV-radiation induced the increased content not only of resveratrol, but also of viniferins in grapevine (Langcake and Pryce, 1976; Langcake and Pryce, 1977a; Langcake, 1981). The reaction from *trans*-resveratrol to its dimer *trans*-ε-viniferin and larger oligomers is an oxidative dimerization, most likely under direction of a peroxidase.

It was shown at the time and again later that the production of a similar substance from resveratrol was possible by using horseradish peroxidase-hydrogen peroxide (Langcake and Pryce, 1977b; Calderón, 1990) or by a laccase from *Botrytis cinerea* (Breuil et al., 1999). Laccases are multicopper oxidases that can be found in plants, fungi and prokaryotes and are also grouped as polyphenol oxidases (Mayer, 2002; Claus, 2003; Hoegger et al., 2006; Aniszewski et al., 2008; Liu et al., 2020). In plants, they differ in their substrate specificity and thus are involved in a wide range of functions, including lignin metabolism (Bourbonnais and Paice, 1990; He et al., 2019) and flavonoid polymerization (Pourcel et al., 2005; Jaiswal et al., 2010).

Table 1: Comparison of the molecular structure of selected stilbenes.

Selected stilbenes produced in different plant species are compared regarding their side chains. Depicted are only the trans forms of each respective molecule. O-Glc: O-β -D-glucopyranoside, TS: 2,3,4',5-tetrahydroxystilben-2-glycosid. Figure adapted and modified from (Chong et al., 2009).

Stilbene	R2'	R3'	R5'	R3''	R4''	R5''	R6''
resveratrol	H	OH	OH	H	OH	H	H
piceid	H	O-Glc	OH	H	OH	H	H
pterostilbene	H	OCH_3	OCH_3	H	OH	H	H
piceatannol	H	OH	OH	H	OH	OH	H
pinosylvin	H	OH	OH	H	H	H	H
oxy-resveratrol	H	OH	OH	H	OH	H	OH
TS	O-Glc	OH	OH	H	OH	H	H
mulberroside	H	OH	O-Glc	H	O-Glc	H	OH
rhaponticin	H	O-Glc	OH	H	O-CH_3	OH	H
rhapontigenin	H	OH	OH	H	O-CH_3	OH	H



1.3.6 Stilbenes in grape berry ripening

The MYB TFs in *V. vinifera* and by extension also stilbene synthases, as well as most likely also the potential modification genes and at least some stilbenes show a change in expression or metabolite levels during the development of the grape berries during the season (Höll et al., 2013). This can be seen for numerous metabolites, primary or secondary.

In grape berries, the three types of tissues are the berry skin, its flesh and the seeds. During the weeks of development, there are two main stages, first the growth phase, followed by the ripening phase. They are divided by a timepoint called véraison. The first phase takes around 60 days, while the second phase may take a few days or even a week less, both phases depend on many factors, including climate and weather conditions, but also treatment by the wine-growers (Coombe, 1992; Ollat et al., 2002). Growth still occurs in the ripening phase, separated from the first weeks of growth by a lag phase (Coombe and McCarthy, 2000). The developmental stages seem to be under control of an interplay between various phytohormones with auxins, cytokinins and gibberrelins present in the beginning of the growth phase and likely regulating amongst other things the switch from cell division to cell expansion. Ethylene, abscisic acid and brassinosteroids then regulate the switch from growth to ripening and the accumulation of various metabolites (Coombe, 2001; Conde et al., 2007). During the growth phase, two important compounds that accumulate are tartaric acid and malic acid, which are important in the acidity of wine. Furthermore, tannins or proanthocyanidins (PAs), which are responsible for astringency in red wines have their highest concentration in this phase and occur in the seeds and grape skin (Kennedy, 2002; Conde et al., 2007) (Figure 2). As representatives of primary metabolism, sugars accumulate from véraison on and are immensely important for wine quality and might furthermore even be involved in regulation of flavonoids (Tsukaya et al., 1991; Jackson and Lombard, 1993). Belonging to the latter class, anthocyanins can mostly be found in the skin of grape berries of the red cultivars and are responsible for the red wine color. Finally, volatile small compounds that are responsible for the different wine aromas, accumulate in the ripening phase (Conde et al., 2007).

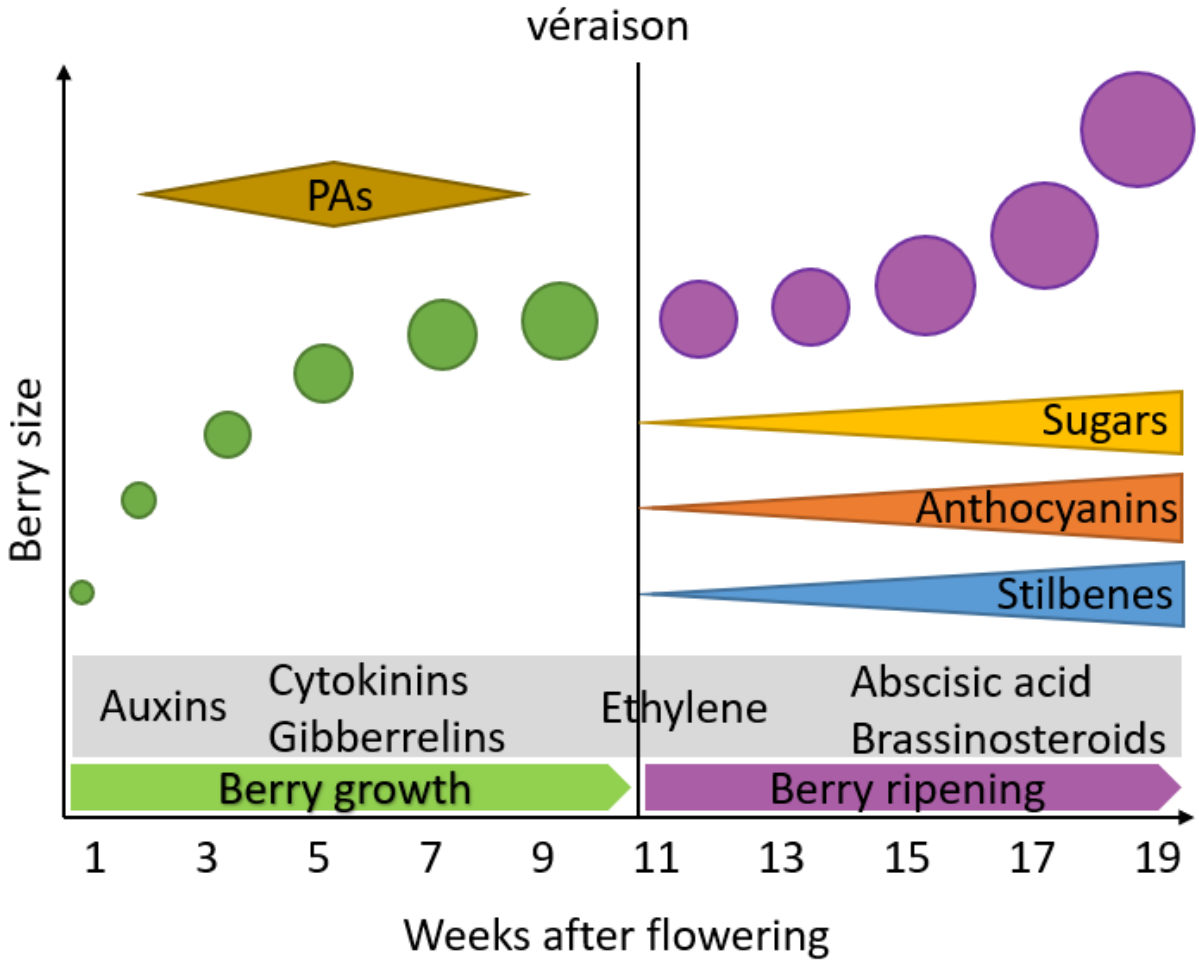


Figure 2: Developmental stages and selected metabolites of *V. vinifera* berries.

The developmental stages of *V. vinifera* berries (red cultivar), growth and ripening, are depicted separated by a lag phase around véraison (week 10-11). The y-axis depicts weeks after flowering, the x-axis berry size. The content of proanthocyanidins (PAs), sugars, anthocyanins and stilbenes as selected metabolites are depicted for a time-wise overview and not relative in amount. Phytohormones are shown in their relative time of regulation. Adapted and modified from Kennedy (2002) and Conde et al. (2007).

1.3.7 Roles of stilbenes in plants

Secondary metabolites that are involved in the responses to stress factors, biotic and abiotic, can be broadly categorized into phytoanticipins or phytoalexins. The term phytoalexin was first coined by Müller (1940) and specified later by Paxton (1980; 1981) according to new insights as being of low molecular weight, antimicrobial compounds which are synthesized and accumulate in plants when exposed to microorganisms. The term phytoanticipins was later defined similarly, but with the distinction of being present in plants before microorganismal contact or as being produced from substances that were present beforehand (VanEtten et al., 1994). Since those definitions are based on

their timepoint of synthesis, their role in defense may well vary from plant to plant, depending on its pool of prepared compounds and induced synthesis upon infection (VanEtten et al., 1994). One function of stilbenes as phytoanticipins, that was discovered very early on was their role in the prevention of wood decay in pines due to their antimicrobial activity (Hart and Shrimpton, 1979; Hart, 1981). Similarly, stilbenes can be found in most parts of the *Vitis* plants, with varying amounts and compositions between different species and cultivars. Here as well, they were found in woody parts and roots, also exhibiting insecticidal functions, for example against the potato beetle (*Leptinotarsa decemlineata*), or against grape phylloxera (*Daktulosphaira vitifoliae*) (Korhammer et al., 1995; Jeandet et al., 2002; Gabaston et al., 2018; Eitle et al., 2019).

While the presence of stilbenes in woody tissues and roots might be promising for stilbene extraction in the future (Gabaston et al., 2017), the economic main focus of grapevine plants is on the berries themselves. Several researchers showed that stilbenes, including *trans*-resveratrol and its glycosylated derivatives like *trans*-piceid, accumulate in grape berries during ripening, even without infection-stress (Jeandet et al., 1991; Versari et al., 2001; Gatto et al., 2008). There they occur in the exocarp along with STS expression, which seems to hint towards their production as phytoanticipins in defense against infection from e.g. fungi that would grow on the berries (Versari et al., 2001; Fornara et al., 2008). Furthermore, it was shown that the accumulation and expression of STS also correlates with the expression of the transcription factors *VvMYB14* and *VvMYB15* (Höll et al., 2013). This accumulation, if not always the gene expression was also shown across several *Vitis vinifera* cultivars and *Vitis* species (Gatto et al., 2008; Kiselev et al., 2017; Gabaston et al., 2020). The absolute amount varies strongly between cultivars, tissues and especially in field experiments between growth conditions, but seems to usually be in a low µg/g freshweight range for trans-resveratrol and trans-piceid in 'Pinot Noir' (Jeandet et al., 1991; Gatto et al., 2008). The amounts of stilbenes, specifically *trans*-resveratrol and *trans*-piceid, in wine also varies considerably between cultivars, regions and years, but is mostly in the single-digit numbers of mg/l, with some outliers below and above (Lamuela-Raventos et al., 1995; Gil-Muñoz et al., 2017; Gocan).

In contrast to these patterns of stilbene accumulation that fit the definition of phytoanticipins, there are also many publications on their importance as phytoalexins. This, again, is not limited to *Vitis* species, but was also discovered in *Pinus sylvestris*. There, seedlings that were subject to *Botrytis cinerea* infection showed a 38-fold higher STS activity than their non-infected counterparts (Gehlert et al., 1990). Over the years, several researchers have connected stilbenes, mainly *trans*-resveratrol, *trans*-viniferin and *trans*-pterostilbene, to infections of grapevine plants, respectively to their increased resistance against the pathogen attack and the toxicity of stilbenes against certain fungi (Langcake and Pryce, 1976; Dercks and Creasy, 1989; Paul et al., 1998; Pezet et al., 2004b; Schnee et al., 2008; Liu et al., 2019). In leaves of grapevine plants, this phytoalexin property is easily observed,

since usually the stilbene amounts are very low, to non-detectable, but increase greatly upon infection. Again, the exact amounts are dependent on previously mentioned factors, but are close to 0 µg/g freshweight in uninfected leaves and between 40 (*trans*-resveratrol) and 2500 µg/g freshweight (total stilbenes) in infected ones (Langcake and Pryce, 1976; Poutaraud et al., 2007). The effectiveness of stilbenes against *P. viticola* infection was tested *in vitro* by analyzing the release and mobility of zoospores. Experiments showed that, while *trans*-resveratrol has a toxic effect, *trans*-viniferin and *trans*-pterostilbene were much more effective against the spores (Langcake and Pryce, 1976; Dercks and Creasy, 1989; Pezet et al., 2004a). In vivo, there is also evidence that stilbene content in plants correlates with improved resistance against *Plasmopara viticola*. In that case, comparisons between resistant and susceptible cultivars revealed increased stilbene levels in the resistant ones (Pezet et al., 2004b; Eisenmann et al., 2019). The same observations were made in leaf material infected with *Erysiphne necator*, responsible for powdery mildew (Schnee et al., 2008) and *in vitro* with *Botrytis cinerea* (Adrian et al., 1997). A special property of some *B. cinerea* strains is their ability to metabolize certain stilbenes, thereby circumventing the defense capabilities of the compounds. This was shown to affect *trans*-resveratrol and *trans*-pterostilbene and to be achieved by a laccase-like enzyme (Sbaghi et al., 1996; Adrian et al., 1998; Breuil et al., 1999). Another defense mechanism of *B. cinerea* against stilbenes, specifically resveratrol is an ABC-transporter that was shown to decrease the sensitivity of the pathogen against resveratrol (Schoonbeek et al., 2001). Besides the anti-fungal and insecticidal properties of stilbenes already mentioned, researchers have also investigated the increase of stilbene content and thus initiation of the defense reaction, in response to bacterial or microbial attack (Verhagen et al., 2011; Hao et al., 2012), although these properties and possible interactions with antifungal effects are beyond the focus of this work.

Besides the role of stilbenes in plant-pathogen interactions or defense reactions, they are said to be active in one more type of interaction: plant-plant allelopathy. Allelochemicals are active in signaling between different organisms, in this case between plants of the same or different species, to inhibit growth and development and thus conserve space or resources for the emitting plant. Stilbenes and flavonoids have shown to be involved in this kind of signaling by inhibiting seed germination and seedling growth (Fiorentino et al., 2008; Li, 2010).

Besides reactions to biotic stresses, the involvement of stilbenes has also been investigated in response to abiotic stress factors, such as UV-C light as well as mechanical injury and treatment with Ozone in *V. vinifera* and/or P. *sylvestris* (Langcake and Pryce, 1976; Chiron et al., 2000b; Pezet et al., 2003; Poutaraud et al., 2007; Suzuki et al., 2015).

1.3.8 Plant defense mechanisms

Whereas the correlation of successful plant defense with the accumulation of stilbenes has been shown sufficiently and even *in vitro* assays have attested to their fungitoxicity, the exact role and mechanisms of stilbenes in these reactions remain to be elucidated.

In general, the totality of plant defense mechanisms have been described as the plant immune system by Jones and Dangl (2006) with their zig-zag model (Figure 3). It is described as a model with two layers, one general system for the recognition of pathogen associated molecular patterns (PAMPs) that are essential for most pathogens, as well as damage associated molecular patterns (DAMPs), leading to pattern triggered immunity (PTI) and one more specialized adapting to specific pathogens and their effectors against the first layer, called effector triggered immunity (ETI), which shows remarkable similarities to animal immune responses.

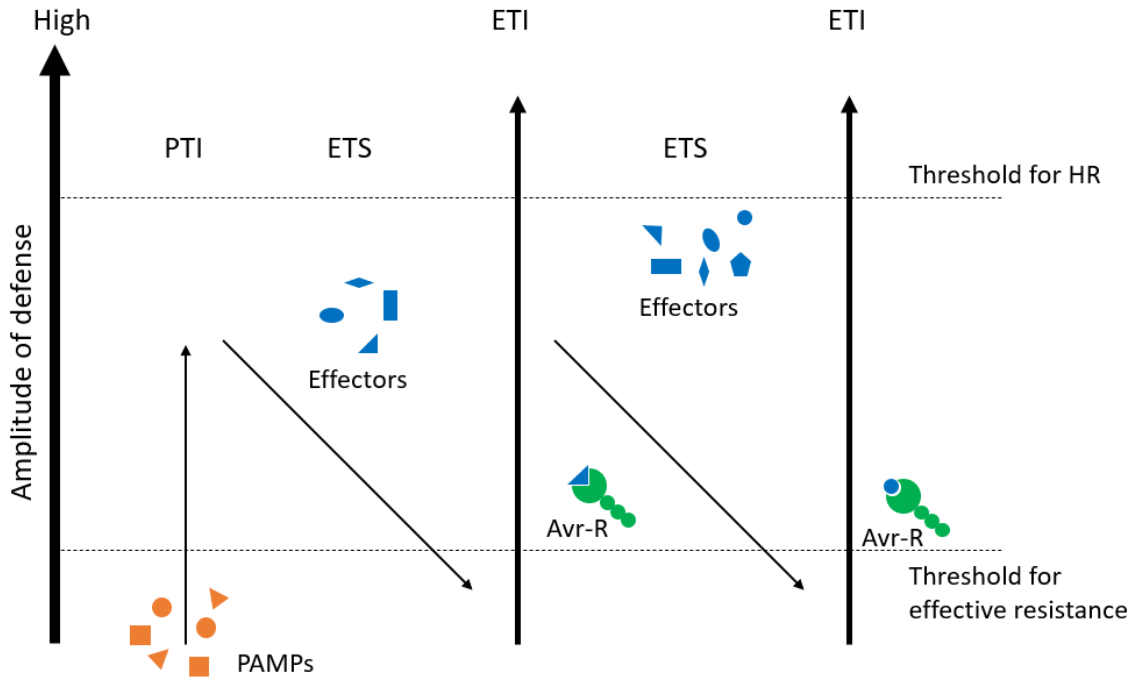


Figure 3: The zig-zag model of the plant immune system after Jones and Dangl (2006).

This model depicts the alternating steps of pathogen attack and plant defense during the interaction of both. Pathogen-associated molecular patterns (PAMPs) are recognized by the plants pattern recognition receptors (PRRs) triggering an immune response, the PAMP-triggered immunity (PTI). In reaction to that, the pathogen deploys effectors designed to disable or prevent PAMP recognition leading to a so-called effector-triggered susceptibility (ETS). The plant can then react to this by developing a resistance protein (R-protein) for recognition and neutralization of the effector, leading to effector-triggered immunity (ETI) and effectively rendering the effector an avirulence factor (Avr). This cycle of effector generation and recognition by new R-proteins can repeat, leading to an arms-race between plant and pathogen. Figure designed according to Jones and Dangl (2006).

This indicates that susceptibility to pathogens and their diseases is caused by the suppression of both layers due to plant-pathogen competition in their co-evolution (Jones and Dangl, 2006; Keller et al., 2016). PTI is triggered by the recognition of molecular patterns common for pathogens, which include flagellin, cold shock proteins of bacteria, elongation factors or more and which are usually difficult to shed or mask for their owners, since they are essential (Felix et al., 1999; Felix and Boller, 2003; Kunze et al., 2004; Jones and Dangl, 2006). Besides recognizing the pathogens directly, DAMPs such as cell wall fragments, ATP and others can also trigger the PTI, since they also ultimately indicate a danger for the cells (Vance et al., 2009; Dodds and Rathjen, 2010). The recognition itself is achieved by so called pattern recognition receptors (PRRs) reaching to the outside of the cell membrane (Dangl et al., 2013). Pathogens can overcome this initial defense with the help of their effectors, which of course vary from pathogen to pathogen, by suppressing the PTI, causing the plant cells to become susceptible (Jones and Dangl, 2006; Keller et al., 2016). This is where the arms race between plant and pathogen begins. As their second defense layer, plants developed specific mechanisms to recognize such effectors and thus trigger ETI. Typical receptors for effector recognition are proteins with nucleotide binding and leucin rich repeat domains (NB-LRR), encoded by resistance genes (R-genes) (Dangl and Jones, 2001; Dodds and Rathjen, 2010). Since the establishment of the model and during further research, it has become evident that sometimes, a clear distinction between PTI and ETI is difficult (Thomma et al., 2011). Nevertheless, once a pathogen is recognized and the response is not suppressed, there are signaling cascades and downstream responses that follow. The signaling cascades are especially not yet well understood, but likely include calcium signaling, mitogen-activated protein kinase (MAPK) cascades, and reactive oxygen species (ROS) (Nürnberger and Scheel, 2001; Dodds and Rathjen, 2010; Seybold et al., 2014). Downstream of this signaling, there are different factors that are affected. One group are the phytohormones, especially jasmonic acid, ethylene and salicylic acid, which seem to act in and coordinate defense responses, depending on their interactions and on what kind of attack exactly occurs (Bari and Jones, 2009; Verma et al., 2016). Another important building block of the plants defense response are so called pathogenesis related-proteins (PRs). They are divided into 17 different families and include a wide variety of enzymes, like chitinases, glucanases, proteinases, peroxidases and more (van Loon et al., 2006). These PR proteins can fulfill a wide variety of functions, e.g. glucanases have been shown to be involved in cold tolerance, development and germination (Yaish et al., 2006; Romero et al., 2008; Balasubramanian et al., 2012) as well as defense responses including direct attacks on pathogen cell walls or indirectly via elicitor release (Somssich and Hahlbrock, 1998; Mohammadi and Karr, 2002). In addition to these, some resistance proteins were discovered that encode ABC (ATP binding cassette) transporters (Stein et al., 2006; Krattinger et al., 2009). ABC-transporters are present in all life forms and consist of a cytosolic domain for ATP hydrolysis and a trans-membrane domain for substrate translocation (Hyde et al., 1990). In *Vitis vinifera*, there are 135

genes reported that code for an ABC-transporter (Çakır and Kılıçkaya, 2013). ABC-transporters have different functions across different organisms, which in plants include flavonoid and anthocyanin transport (Goodman et al., 2004; Braidot et al., 2008; Francisco et al., 2013) and act in pathogen resistance (Stein et al., 2006; Krattinger et al., 2009). As a last resort in defense, there is programed cell death (PCD), which is especially effective against biotrophic pathogens like *P. viticola*. It involves a burst of reactive oxygen species, although the exact signaling and mechanisms leading up to it remain to be discovered (Williams and Dickman, 2008; De Pinto et al., 2012). Often defense reactions in plant tissue, especially those triggered by ETI, lead to a hypersensitive response, which can consist of one or several of the mechanisms mentioned before and may not only be involved in the direct mechanisms but also in long range signaling in the plant and the consequential priming of the plant against secondary infection (Alvarez et al., 1998). In this balance of reactions, stilbenes might not only have their roles as defense compounds due to their antifungal and other properties, but also act in the control of ROS. Since not all defense situations call for ROS induced cell death, the damage has to be prevented by antioxidant molecules, to which the stilbenes can be counted due to their free radical scavenging properties. Modified stilbenes might even be especially efficient in these roles, compared with the simpler resveratrol (Privat et al., 2002; Apel and Hirt, 2004; Mittler et al., 2004; Mikulski and Molski, 2010).

Among the pathogens that plague grapevine plants in the vineyards, *Plasmopara viticola* and the disease caused by it, downy mildew, has been the focus of attention due to its being widespread in the German and middle European vineyards. It has been shown that different species within the *Vitis* family, especially from north America and Asia, show different levels of resistance to *P. viticola*, which has been connected to various loci containing a resistance trait, called “Resistance to *Plasmopara viticola*-loci” (Rpv) (Cadle-Davidson, 2008; Díez-Navajas et al., 2008; Possamai et al., 2020). So far, 31 of these loci are known (Possamai et al., 2020; Sargolzaei et al., 2020; Vitis International Variety Catalogue VIVC, 2020), including two versions of Rpv3 (Rpv3.1 and Rpv3.2) from north American species (Bellin et al., 2009; Di Gaspero et al., 2012; van Heerden et al., 2014), Rpv10 (Schwander et al., 2012) and Rpv12 (Venuti et al., 2013), both from Asian species. Since these single resistance loci can be broken by *P. viticola* strains (Peressotti et al., 2010; Eisenmann et al., 2019), researchers have recently worked towards pyramiding different Rpv loci in order to establish further barriers within one *Vitis* cultivar against such resistant *Plasmopara* strains (Schwander et al., 2012; Zini et al., 2019).

1.3.9 Benefits of stilbenes for humans

Stilbenes, while doubtlessly very beneficial for the plants that produce them, have also been under intense investigation of their potential beneficial health effects for humans in recent years. This was likely initiated by a report on the so-called French paradox. The researchers at the time were investigating the fact that despite of a diet rich in saturated fat, the French people had a remarkably low rate of coronary heart diseases, compared to other industrial nations, which they attributed this to the higher consumption of red wines (Renaud and de Lorgeril, 1992). Although this observation could have many causes, from components of the wine, their interplay, or due to unrelated factors, it sparked many studies, among them the first on resveratrol and its cancer chemopreventive activity (Jang, 1997), with many more following after. As several reviews have pointed out, there are thousands of publications on the health benefits of resveratrol, but very few clinical trials in humans so far (Vang et al., 2011; Berman et al., 2017). Animal trials attest resveratrol has many preventive or healing abilities, which of course have to be viewed with caution until proper clinical studies have confirmed these results in humans. Nevertheless, the field seems promising for future research.

Very essential in any proposed prevention or treatment of diseases by resveratrol are its anti-oxidative and anti-inflammatory properties of resveratrol (de la Lastra and Villegas, 2007; Gülçin, 2010; Bigagli et al., 2017). These basic properties are supported by many animal studies and some clinical indications that suggest that resveratrol helps in prevention or treatment of other important diseases such as cardiovascular diseases (Hung, 2000; Robich et al., 2010), obesity (Lagouge et al., 2006; Dal-Pan et al., 2010), diabetes (Palsamy and Subramanian, 2008; Bhatt et al., 2012) and different types of cancer (Jang, 1997; Savouret and Quesne, 2002). Furthermore, it has been shown to have neuroprotective properties (including in Alzheimers disease) (Ates et al., 2007; Kim et al., 2007) and promote longevity (Valenzano et al., 2006; Bass et al., 2007). The dosage of resveratrol in these experiments varies substantially and conversion into human equivalent doses of course is only an estimate that has to be validated in clinical experiments. The calculated doses would be in a 1 mg – 1 g per day range for an average human (Reagan-Shaw et al., 2008; Vang et al., 2011). So far no adverse effects of resveratrol intake in humans have been reported, beside gastrointestinal discomfort or diarrhea when several grams of resveratrol were ingested per day (Vang et al., 2011).

Resveratrol and wine compounds in general have not only received the attention of the medical community as treatments or food supplements, but also from the cosmetic industry, mainly due to the mentioned UV-protective and anti-aging-functions that can be used in marketing as natural and beneficial ingredients, as reviewed by Soto and colleagues (Soto et al., 2015).

While it is possible to extract stilbenes from plant tissue, especially grape berries, artificial production by microorganisms or cell culture has also been established, especially if pure resveratrol is desired. There have been several attempts to genetically modify microorganisms by introducing genes from stilbene producing plants in order to have the microorganisms produce the desired resveratrol. The two main strategies here are to either transform the whole needed pathway, so that the microorganism can produce resveratrol form its own amino acids as substrate, or secondly to only transform STS and coumaroyl-CoA ligase and feed the respective precursor as substrate in the medium (Donnez et al., 2009). There have been successes for the feeding approach in bacteria and yeast, although optimization was needed and will still be needed to increase the very low amounts produced (Becker et al., 2003; Beekwilder et al., 2006; Zhang et al., 2006). The entire-pathway-transformation approach yielded successful strains as well, although again with future optimization and work needed (Vannelli et al., 2007; Xue et al., 2007). Of these, one patented strain was obtained for use in this project (Katz et al., 2013).

Plants are another common way to obtain resveratrol and Japanese Knotweed (Polygonum cuspidatum) is one frequent source of resveratrol of varying purity, depending on the processing (Donnez et al., 2009). Since other plants, such as grapevine or pines are poorly suited for industrial scale stilbene production because of their slow growth, other plant-based systems have been considered. The production of stilbenes has been achieved for example in grapevine cell suspension culture, where it can be greatly increased beyond the natural levels by using elicitors (e.g. methyl jasmonate) of stress or defense reactions (Larronde et al., 1998; Repka et al., 2004; Tassoni et al., 2005; Martínez-Márquez et al., 2016). Furthermore, resveratrol production in hairy root culture or callus culture from either a stilbene producing plant or a transformed plant such as tobacco can be utilized (Guillon et al., 2006; Donnez et al., 2009; Hidalgo et al., 2017).

Hairy root cultures, agrobacterium-transformed grapevine plants and transiently transformed plants have been used before in the work group during the work with the MYB transcription factors VvMYB14 and VvMYB15 and were available as basis for this project (Höll, 2014).

1.4 Project goals

Due to the importance of stilbenes for plant defense, especially in *Vitis vinifera*, as well as its potential benefit for human consumption or application against various maladies, it is essential to know the exact biosynthetic pathway, including enzymes, transporters and regulation mechanisms. The elucidation of this biosynthetic pathway beyond the stilbene synthase as first dedicated enzyme was started by the identification and characterization of a resveratrol-o-methyltransferase (Schmidlin et al., 2008) as well as the two main transcription factors VvMYB14 and VvMYB15 (Höll et al., 2013). Using this knowledge, especially the two transcription factors, this thesis aimed at the identification and characterization of further genes that encode either enzymes for stilbene modification or transporters. The basis of this project were the microarrays, conducted by Dr. Janine Höll, which provided data on the differences in transcript levels upon overexpression of VvMYB15, thus providing a large group of genes of which to select our so-called candidate genes. From there on, this study was divided into three main parts, all including several sets of experiments and analysis.

First, the suitable candidate genes were to be identified from the list of potential VvMYB15 targets and their affiliation with the VvMYB15 regulatory network was to be investigated.

Second, promising candidate genes under the control of VvMYB15 were subjected to investigations of correlation between candidate gene expression and stilbene content, in order to not only confirm an association of the gene with an expected modified stilbene, but furthermore place the gene and potential product in a context of function within the plant.

Third, and finally, the most promising candidate genes were analyzed biochemically for their ability to directly produce a modified stilbene in an enzymatic assay.

The complete chain from identification to characterization of a candidate gene would then allow either for biotechnological production of a specific, pure stilbene, e.g. via yeast cultures, or, depending on its *in-planta*-function, selective breeding for more resistant, and hopefully good-tasting, *Vitis vinifera* cultivars, although both are of course rather long term goals.

2 Results

2.1 Identification of candidate genes by DNA microarray analysis of VvMYB15 overexpressing tissue

2.1.1 Expression levels of selected candidate genes in *V. vinifera* were increased up to 8-fold after ectopical expression of *VvMYB15*

Previous findings of Dr. Janine Höll et al. (2013, 2014) revealed that two transcription factors (TFs), VvMYB14 and VvMYB15, are regulators of stilbene biosynthesis in *V. vinifera*. Based on this information, a non-targeted approach was used in order to identify genes with potential to modify or transport stilbenes downstream of those transcription factors. Therefore, three experiments resulting in two separate microarrays were performed by Dr. Janine Höll et al. (2013, 2014) of which the data were used as basis of this project. *VvMYB15* overexpressing grapevine plants (Selektion Oppenheim 4) were generated and along with *VvMYB15* overexpressing grapevine ('Chardonnay') hairy roots used for a first microarray analysis. The second one was performed with leaf tissue (*V. vinifera* cv. 'Shiraz') that was transiently expressing *VvMYB15* due to agrobacterium mediated transformation. The results of both are displayed in Table 2 as fold changes of control tissue (untransformed or GFP control). In both microarrays, *VvMYB15* showed an increase of 44.62 or, 31.55-fold higher expression that the controls (Microarray A) and 9.93-fold increased expression (Microarray B). In both arrays, several *STS* genes were overexpressed (data not shown), although here, only *VvSTS29* (representing *VvSTS25*, *VvSTS27* and *VvSTS29*, which cannot be distinguished by the primers (Höll, 2014)) displayed, since it was used as representative of the gene family in this project. It is 15.84-fold higher expressed in microarray B than in the control. Furthermore, the candidate genes *VvGT2*, *VvGT4*, *VvLAC*, *VvABC12*, *VvABC13*, *VvStOXY1*, *VvStOXY2*, *VvGLUC1* and *VvGLUC2* were found with increased expression in Microarray A, while *VvGT5*, *VvGLUC1*, *VvGLUC2* and the already published gene *VvROMT* (Schmidlin et al., 2008) were found with higher expression in Microarray B.

Table 2: Relative expression levels of selected V. vinifera genes in VvMYB15 overexpressing tissues measured via microarray analysis.

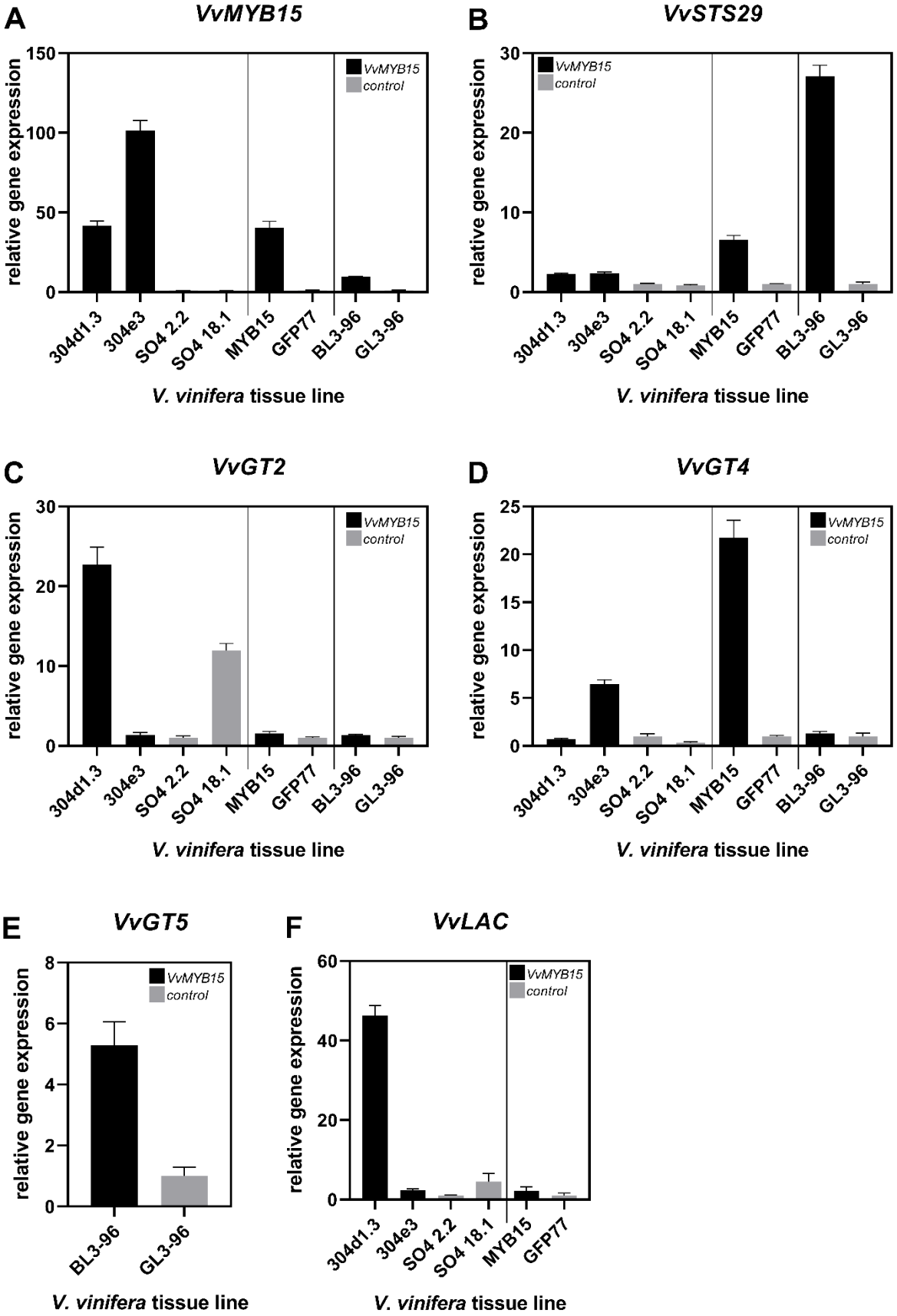
Two separate microarrays were performed. Microarray A with *V. vinifera cv. 'SO4'* leaves from plants ectopically expressing *VvMYB15* (pEX13:VvMYB15; untransformed plants as control) as well as tissue from *V. vinifera* cv. 'Chardonnay' hairy root culture ectopically expressing *VvMYB15* (pART27:VvMYB15; GFP-expressing plants as control) and Microarray B with leaves from *V. vinifera* cv. 'Shiraz' that were transformed with pKGWFS7:VvMYB15 or an empty control vector via agrobacterium mediated infiltration. Displayed are manually chosen candidate genes with their VIT-numbers for identification and fold expression changes. Microarray A values were calculated as mean values of the combined lines SO4-2.2, SO4-18.1 and GFP-77 (two untransformed plant lines and one GFP-control hairy root line)) and the mean value of the combined lines 304d1.3, 304e and MYBB2-2 (two VvMYB15-expressing plant lines and one VvMYB15 expressing hairy root line). Microarray B values represent the expression between one control and one transformed plant 96 hours after infiltration.

Gene	Vit numbers	Fold change microarray A	Fold change microarray B
VvMYB15 (probe1)	VIT_16s0100g00990	44.62	x
VvMYB15 (probe2)	VIT_09s0002g05570	31.55	x
VvMYB15 (probe3)	VIT_05s0049g01020	x	9.93
VvMYB14	VIT_05s0049g01020	x	22.78
VvSTS29	VIT_05s0049g01020	x	15.84
VvGT2	VIT_03s0180g00200	2.83	x
VvGT4	VIT_02s0025g01240	5.13	x
VvGT5	VIT_03s0017g02110	x	4.20
VvLAC	VIT_18s0001g00680	2.42	x
VvABC12	VIT_07s0005g02660	2.15	x
VvABC13	VIT_18s0001g11470	2.85	x
VvStOXY1	VIT_18s0001g11430	7.75	x
VvStOXY2	VIT_06s0061g00120	7.05	x
VvGLUC1	VIT_05s0077g01150	8.15	8.23
VvGLUC2	VIT_03s0180g00200	3.11	7.28
VvROMT	VIT_12s0028g01880	x	7.73

2.1.2 Gene expression levels of *VvMYB15*, *STS29* and candidate genes were confirmed by measurements via quantitative real time PCR

Since DNA-microarray experiments are known to give false positive results, it is recommended to evaluate the expression of genes of interest and confirm their changed expression levels by quantitative real time PCR (qPCR).

Two microarrays were analyzed, comprising three experiments. The material for the first array were *V. vinifera* cv. 'SO4' plants as well as grapevine hairy root lines (cv. 'Chardonnay'), both expressing either *VvMYB15* or being empty controls (plant lines) or transformed with *GFP* (hairy roots). The second array was performed on *V. vinifera* cv. 'Shiraz' leaves that were transiently expressing *VvMYB15* or *GFP*. Figure 4 depicts the expression levels of selected genes. *VvMYB15* showed increased expression levels in comparison to the control lines in all three experiments, ranging between 100-fold in line 304e3 and 9.5-fold in sample BL3-96 (Figure 4 A). *VvSTS29*, the first stilbene-specific gene, and therefore a good positive control for upregulation by the VvMYB15 transcription factor, reached expression levels of approx. 2.3-fold in the transformed plant lines, 6.5-fold in the hairy roots and 27-fold higher expression than the control in the leaf infiltration (Figure 4 B). The glycosyltransferases (*VvGT2*, *VvGT4* and *VvGT5*) showed some variation between them. *VvGT2* (Figure 4 C) is expressed at increased levels in the plant lines 304d1.3 and SO4 18.1, which is one of the control lines, *VvGT4* (Figure 4D) shows increased expression in lines 304e3 and MYB15 and VvGT5 (Figure 4 E) was only measured in the leaf infiltration tissues of the second array, where it shows ca. 5-fold increased expression when compared to the control. The laccase (*VvLAC*, Figure 4 F) that was chosen for investigation showed expression levels that were 46-fold increased as compared with the respective control in the plant line 304d1.3. In addition to these enzymes, there were also two ABC-transporters (*VvABC12* and *VvABC13*) measured. While *VvABC12* was increasingly expressed mainly in line 304e3 (6-fold) and sample BL3-96 (25-fold), *VvABC13* showed increased expression in line MYB15 (6-fold) (Figure 4 G-H). Finally, also *VvPPO*, a polyphenoloxidase, *VvROMT*, the already known methyltransferase and VvMYB14 showed increased expression in some lines (Figure 4 I-K).


A
VvMYB15
relative gene expression
150
100
50
0
VvMYB15
control
304d1.3
304e3
SO4 2.2
SO4 18.1
MYB15
GFP77
BL3-96
GL3-96
V. vinifera tissue line
B
VvSTS29
relative gene expression
30
20
10
0
VvMYB15
control
304d1.3
304e3
SO4 2.2
SO4 18.1
MYB15
GFP77
BL3-96
GL3-96
V. vinifera tissue line
C
VvGT2
relative gene expression
30
20
10
0
VvMYB15
control
304d1.3
304e3
SO4 2.2
SO4 18.1
MYB15
GFP77
BL3-96
GL3-96
V. vinifera tissue line
D
VvGT4
relative gene expression
25
20
15
10
5
0
VvMYB15
control
304d1.3
304e3
SO4 2.2
SO4 18.1
MYB15
GFP77
BL3-96
GL3-96
V. vinifera tissue line
E
VvGT5
relative gene expression
8
6
4
2
0
VvMYB15
control
BL3-96
GL3-96
V. vinifera tissue line
F
VvLAC
relative gene expression
60
40
20
0
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control
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304e3
SO4 2.2
SO4 18.1
MYB15
GFP77
V. vinifera tissue line

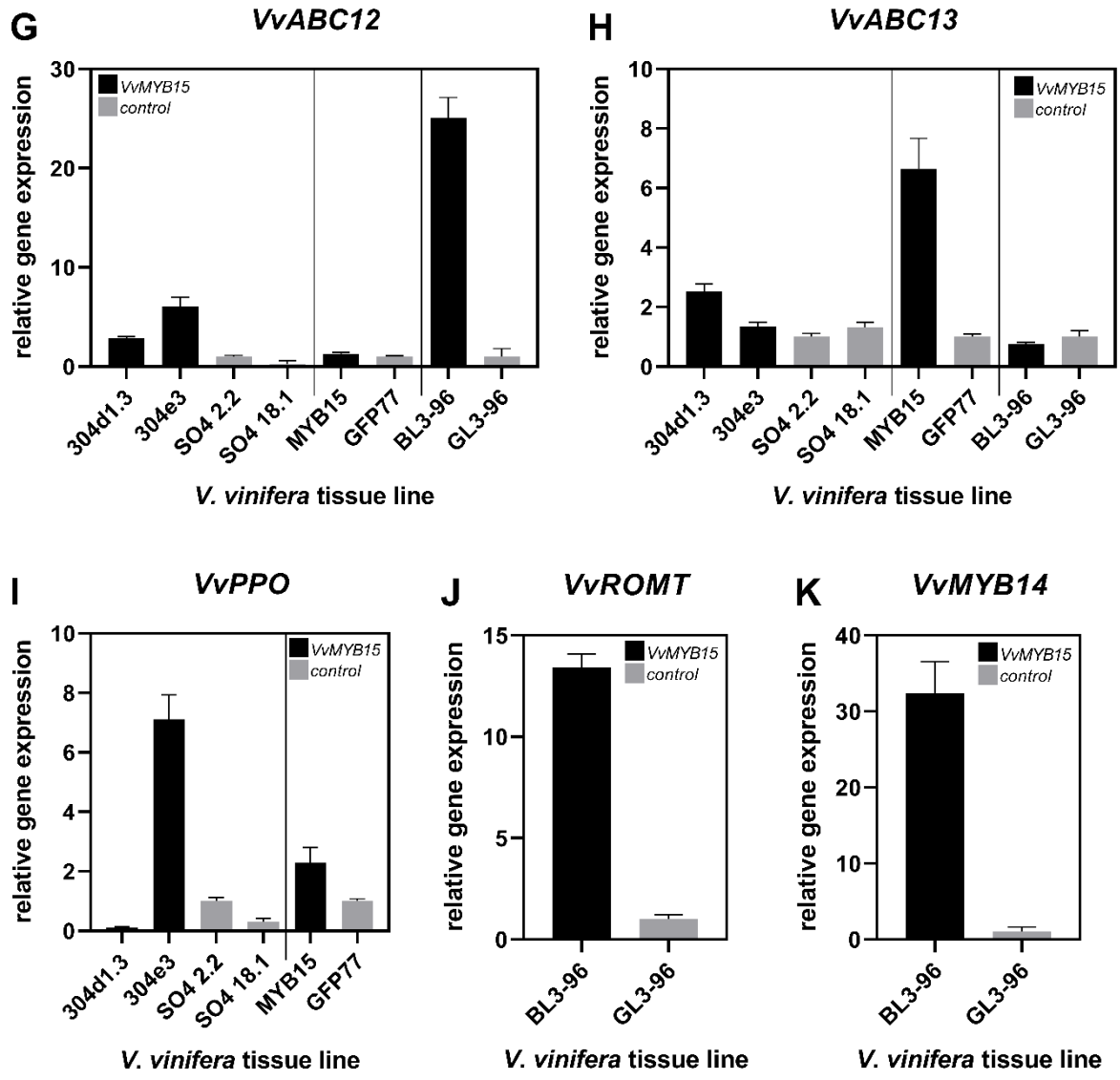


Figure 4: Expression levels of *VvMYB15*, *STS29* and selected candidate genes in different *V. vinifera* tissues ectopically expressing *VvMYB15*.

Expression levels of *VvMYB15* (A), *VvSTS29* (B), *VvGT2* (C), *VvGT4* (D), *VvGT5* (E), *VvLAC* (F), *VvABC12* (G), *VvABC13* (H), *VvPPO* (I), *VvROMT* (J) and *VvMYB14* (K) in *V. vinifera* cv. SO4 plants (left), hairy root lines (cv. 'Chardonnay', center) and *V. vinifera* cv. 'Shiraz' leaves (right), all overexpressing *VvMYB15* or being untransformed/GFP controls. Different tissues are separated by vertical lines. The *V. vinifera* cv. 'SO4' plants were generated from an embryogenetic suspension cell culture by *A. tumefaciens* mediated transformation with *VvMYB15*-pEX13 (35S::VvMYB15, lines 304d1.3 and 304e3). As control, 'SO4' plants were used that were generated from a non-transformed embryogenetic suspension cell culture (lines SO4 2.2 and SO4 18.1). The hairy root lines were generated from *V. vinifera*, cv. 'Chardonnay' by *A. rhizogenes* mediated transformation with pART27:VvMYB15 (line MYBB2-2) or pART27:GFP (line GFP-77) as control line. The *V. vinifera* cv. 'Shiraz' leaves were infiltrated with either *VvMYB15* or *GFP* as control. The samples of the 'Shiraz' leaves depicted were taken after 96 h from plant No.3.

(Figure 4 continued) The expression levels were normalized against the housekeeping genes *VvGADPH*, *VvEF1α* and *VvUBI*, expressed as mean values of one or two replicate PCRs (n=3 or n=6) and error bars indicating SEM. All expression levels are relative to the control line SO4 2.2 for the plant lines, to the control line GFP-77 for the hairy root cultures or to the GFP control in case of infiltration and thus cannot be directly compared between the tissues.

2.1.3 An inducible *VvMYB15* expression vector in stable transformed grapevine plants for future identification of additional candidate genes

It was observed that the *VvMYB15*-transformed plants (*V. vinifera* cv. 'SO4') that showed high expression levels developed stress symptoms and died, thus plant lines with medium expression levels of *VvMYB15* that were able to survive had to be chosen for the microarray experiment. In order to prevent this problem in the future and use the desired high expression levels of *VvMYB15* for the experiment, possibly leading to the discovery of additional candidate genes, Dr. Janine Höll designed dexamethasone inducible constructs. The *VvMYB14* and *VvMYB15* sequences were cloned from 'Pinot Noir' and the Greengate cloning system (Lampropoulos et al., 2013) was used to construct the final Ti-plasmids. They contained modules with the following properties in a Z003 vector backbone: UBQ10 promoter, N-terminal mCherry, glucocorticoid receptor, TEV cleavage site, ORF (or C-dummy C087 in control), 3x myc-tag with stop codon, terminator, hygromycin resistance. Since the transformation into embryogenic grapevine cell culture is very difficult and time consuming, the constructs were, in this project, first re-sequenced and then infiltrated into tobacco in order to confirm their functionality by analyzing the correct localization of the mCherry signal. In two separate infiltration experiments, an *A. thaliana* positive control (AtVHA-a1-mCherry, (Lupanga et al., 2020)) showed the best results in respect to the strength, clarity and localization of the mCherry signal (Figure 5 A, C, E). It could be seen that the localization of the construct at the endoplasmatic reticulum is clearly distinct from the background fluorescence of the chlorophyll. Of the two TFs and the control, *VvMYB15* (Figure 5 B, D, F) showed the strongest signal, but still, the signal was very weak, if at all separable from the background, and the signal also did not perceivably change from the cytosol into the nucleus upon dexamethasone induction.

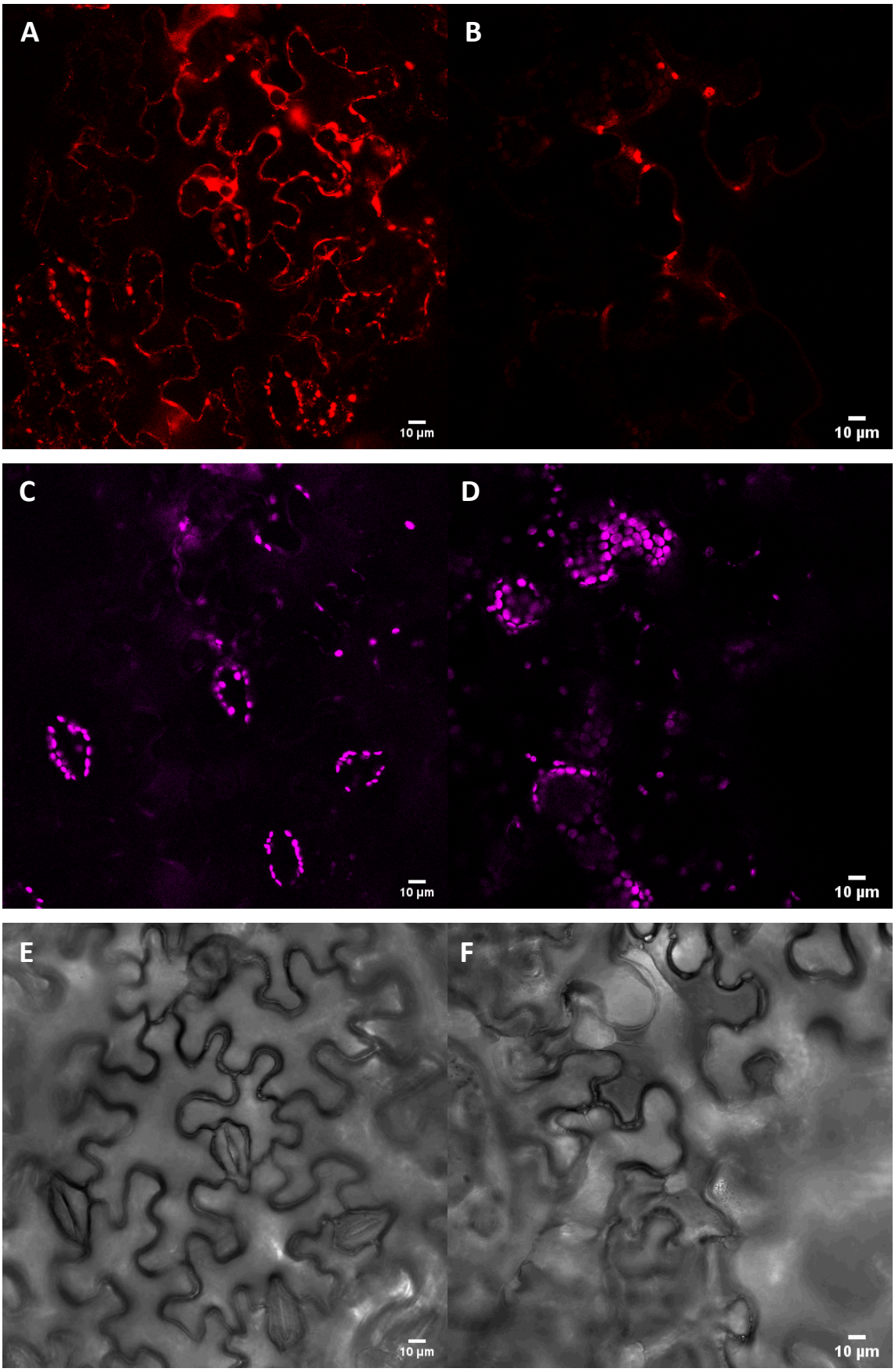
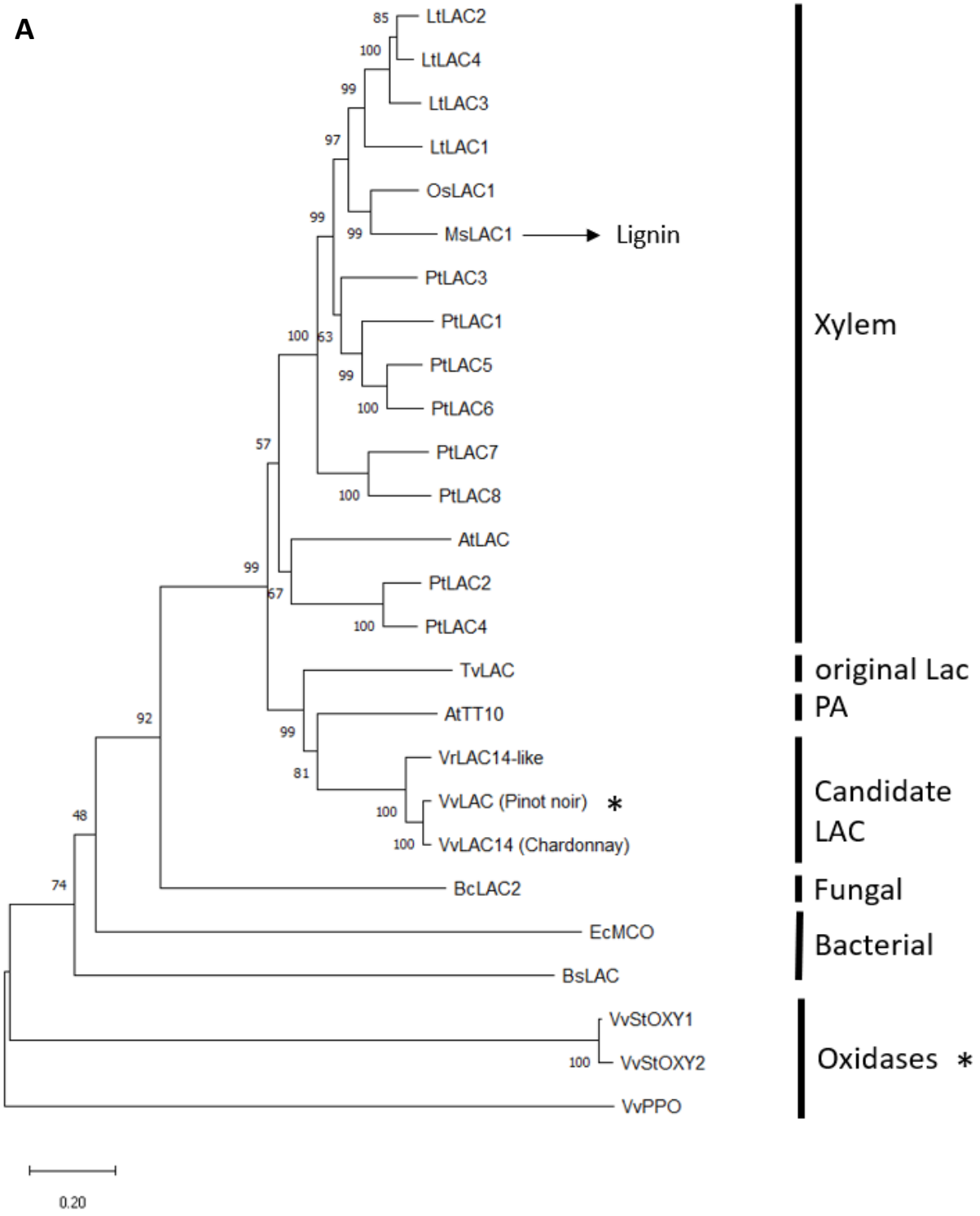


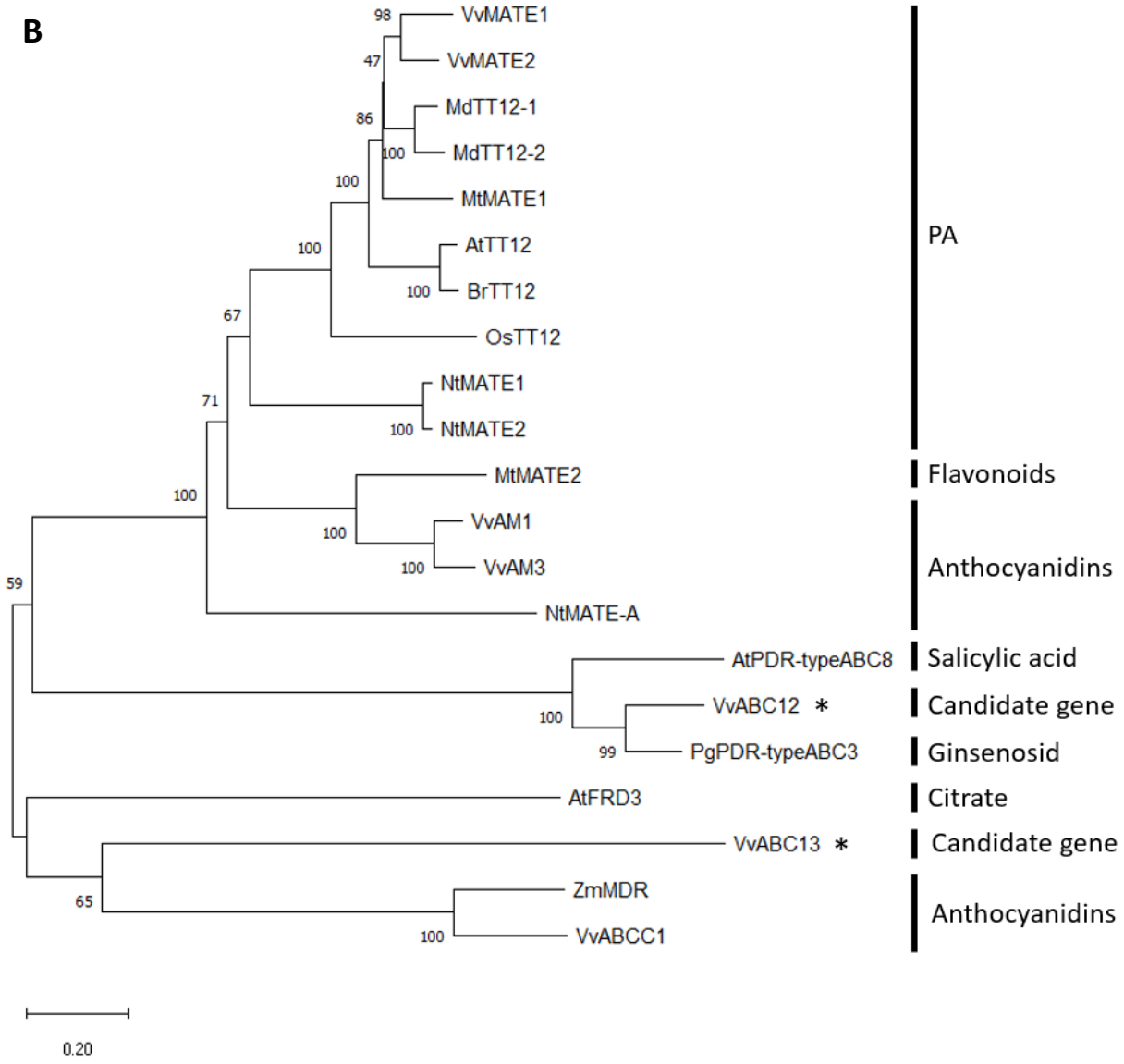
Figure 5: Fluorescence microscopy images of VvMYB15 and AtVHA-a1-mCherry in *Nicotiana benthamiana*. VvMYB15 (B, D, F) and *Arabidopsis thaliana* VHA-a1-mCherry (A, C, E) were infiltrated into 4-week-old *Nicotiana benthamiana* leaves and given three days for the expression of the constructs. Images A and B depict the mCherry

(Figure 5 continued) signal, C and D the chlorophyll background and E and F the white-light image. The localization of VvMYB15 was expected to be in the cytosol and nucleus, since the images are of dexamethasone induced samples and AtVHA-a1-mCherry should be located in the endoplasmatic reticulum and trans-golgi-network in tobacco.

2.1.4 Phylogenetic analysis of laccases and oxidases revealed similarity of the candidate laccase to other polyphenol processing laccases

When working with genes of unknown functions, a comparison with already known or at least predicted genes of similar structure is helpful. This was done in form of phylogenetic analysis for three candidate gene groups: laccases and oxidases (Figure 6 A), ABC-transporters (Figure 6 B) and glycoslytransferases (Figure 6 C). The analysis of laccases shows that VvLAC groups with AtTT10 (transparent testa)(Pourcel et al., 2005), which was shown to be involved in proanthocyanin metabolism as well as the first laccase that was ever extracted by wounding plants (Figure 6 A). This group is opposed by many laccases from different species that are working in the xylem and one specifically with lignin (MsLAC1)(He et al., 2019). Furthermore, a fungal laccase that can metabolize resveratrol (Schouten et al., 2002) is closer related to the candidate gene than two bacterial enzymes. The candidates VvStOXY1 and 2 (putative stilbene oxidases) and VvPPO (polyphenol oxidase) seem to be more distantly related and not directly in the laccase group. The ABC-transporters and related proteins are depicted in Figure 6 B. VvABC13 groups with two anthocyandin related enzymes (Goodman et al., 2004; Francisco et al., 2013), although not that closely, while VvABC12 forms a group with a putative ginsenoside (Zhang et al., 2013) and a salicylic acid transporter (Stein et al., 2006) and more distantly to other flavonol transporters. The glycosyltransferase genes (Figure 6 C) show no clear trend among the enzymes analyzed. The substrates are mostly not definitely shown or the enzymes are possibly able to utilize several substrates. The three candidate genes do not group together. VvGT2 forms a group with several GTs from a set of previously known publications.


A
LtLAC2
LtLAC4
LtLAC3
LtLAC1
OsLAC1
MsLAC1
Lignin
PtLAC3
PtLAC1
PtLAC5
PtLAC6
PtLAC7
PtLAC8
AtLAC
PtLAC2
PtLAC4
TvLAC
AtTT10
VrLAC14-like
VvLAC (Pinot noir) *
VvLAC14 (Chardonnay)
BcLAC2
EcMCO
BsLAC
VvStOXY1
VvStOXY2
VvPPO
0.20
Xylem
original Lac
PA
Candidate LAC
Fungal
Bacterial
Oxidases *


B
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VvMATE2
MdTT12-1
MdTT12-2
MtMATE1
AtTT12
BrTT12
OsTT12
NtMATE1
NtMATE2
MtMATE2
VvAM1
VvAM3
NtMATE-A
AtPDR-typeABC8
VvABC12 *
PgPDR-typeABC3
AtFRD3
VvABC13 *
ZmMDR
VvABCC1
98
47
86
100
100
100
100
67
71
100
100
100
59
100
99
65
100
0.20
PA
Flavonoids
Anthocyanidins
Salicylic acid
Candidate gene
Ginsenosid
Citrate
Candidate gene
Anthocyanidins

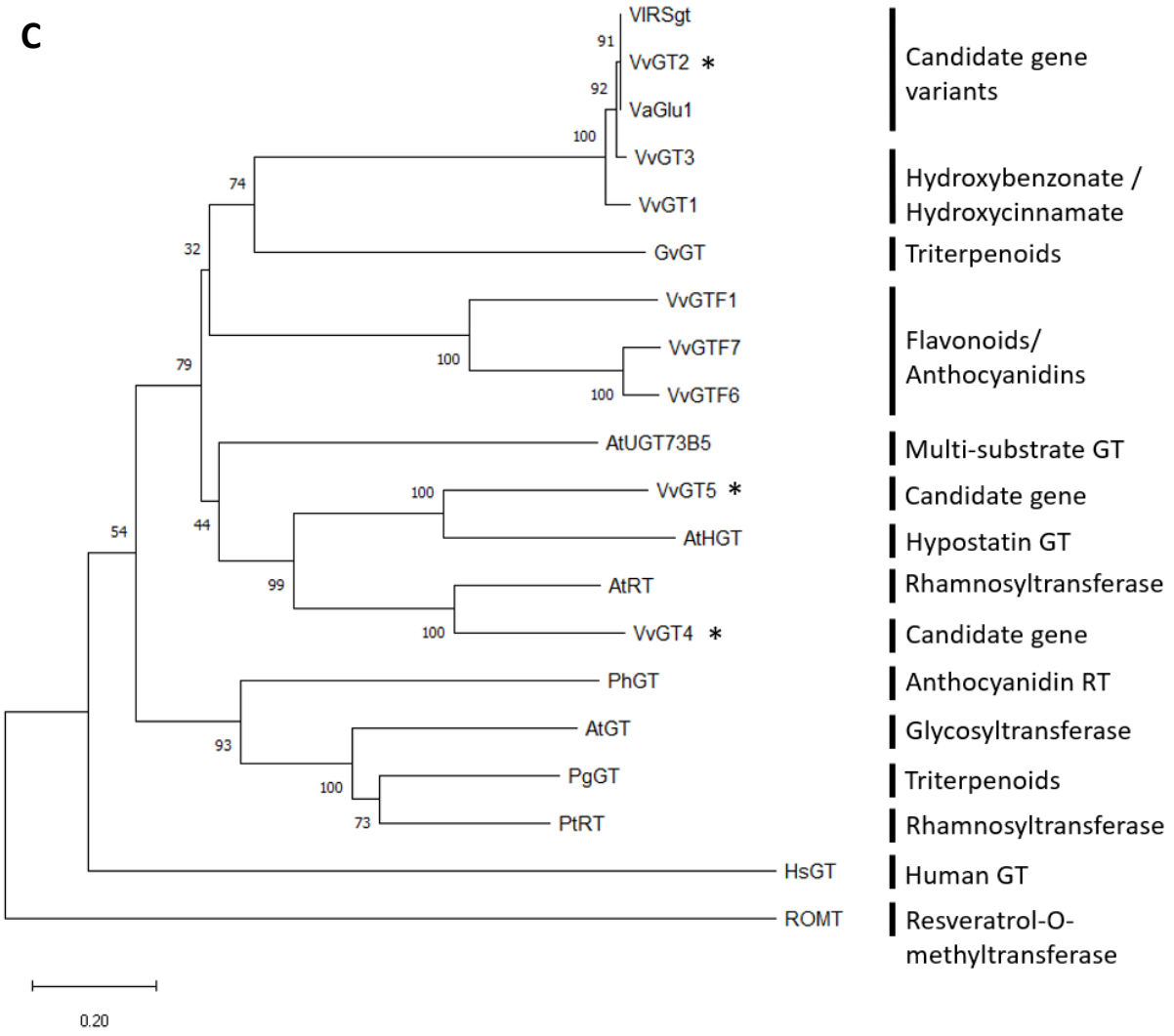


Figure 6: Phylogenetic analysis of candidate genes

The phylogenetic relationship of the protein sequences of the candidate genes (marked "*") with other known genes from their enzymatic-function groups were analyzed by alignment with ClustalW and subsequent creation of a neighbor-joining-tree (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 10.23991884 (A), = 8.83232193 (B), = 9.51279762 (C) is shown. The alignments, calculations and a bootstrap test (10,000 replicates) (Felsenstein, 1985) were done with the MEGA X software (Kumar et al., 2018). Three groups are displayed: laccases (A), ABC-transporters (B) and glycosyltransferases (C). The candidate genes from the microarray analysis are marked with asterisks and published or predicted data on the respective genes are given in the right columns. For more details, including all accession numbers and species abbreviations see material and methods.

2.1.5 Protein localization prediction placed VvMYB15 in the nucleus, VvSTS29 in the cytosol and the ABC-transporters at the plasma membrane

In order to gain insight into the subcellular localization and thus possible hints towards function or substrates of the identified candidate genes, the protein sequences were analyzed with the WoLF PSORT protein localization predictor and the results are presented in Table 3. VvMYB15 was predicted to be most likely localized in the nucleus with a score of 14. This score indicates the number of nearest neighbors to the query which localize to the corresponding site, adjusted by calculations of the logarithm (Horton et al., 2007). For most of the other protein sequences that were analyzed, there was more than one likely option. While VvSTS29 was still more likely to be localized in the cytosol, with a score of 7, than in the chloroplast (score of 3), other genes, including the glycosyltransferases, the laccase and others showed less distinction in the likelihood of localization. A notable exception were the two ABC-transporters (VvABC12 and VvABC13), which seemed to be clearly located at the plasma membrane (scores of 12 and 9, respectively).

Table 3: Subcellular localization prediction of VvMYB15, VvSTS29 and selected candidate gene-products from *V. vinifera* cv. 'Pinot Noir'.

The WoLF PSORT protein localization predictor was used to analyze the likely localization of the proteins encoded by the candidate genes within the cellular compartments. Their names are given alongside the most likely compartment or two compartments if there wasn't one clearly outstanding score. The given score indicates the number of nearest neighbors to the query which localize to each site, adjusted by calculations of the logarithm as described in Horton et al. (2007).

Protein	Most likely compartment	Score
VvMYB15	Nucleus	14
VvSTS29	Cytosol Chloroplast	7 3
VvGT2	Chloroplast	13
VvGT4	Cytosol Chloroplast	8 4
VvGT5	Endoplasmic reticulum Cytosol	5 3
VvLAC	Chloroplast Extracellular space	7 4

Protein	Most likely compartment	Score
VvROMT	Cytosol Cytoskeleton	5.5 5
VvABC12	Plasma membrane	12
VvABC13	Plasma membrane	9
VvGLUC1	Chloroplast Vacuole	8 3
VvGLUC2	Extracellular space Chloroplast	4 3
VvPPO	Chloroplast Chloroplast /Mitochondrion	11.5 7.33
VvStoxy 1	Plasma membrane Endoplasmic reticulum	6 4
VvStoxy 2	Chloroplast	11

2.1.6 VvMYB15 induced the promoters of *VvSTS29*, the glycosyltransferases, *VvLAC*, *VvABC12* and the glucanases

After identification of candidate genes that showed elevated expression levels upon *VvMYB15* overexpression, it was investigated whether the transcription factors VvMYB14, VvMYB15 as well as an anthocyanidin MYB TF increase the induction as compared to a control without added transcription factors. A *Vitis vinifera* cv. 'Chardonnay' cell culture was utilized for particle gun bombardment and subsequent dual luciferase assay in order to show the induction of the promoters corrected for background fluorescence and relative to the respective empty control.

VvSTS29 as first dedicated gene in the stilbene biosynthesis pathway and target of VvMYB15 showed an over 5-fold induction, as has been shown before in Dr. Janine Hölls thesis (Höll, 2014). This again confirmed the function of VvMYB15 to switch on the first step in the stilbene biosynthesis pathway (Figure 7 A). *VvGT4* and *VvLAC* also showed an induction by VvMYB15, as well as VvMYB14, of 3 to 5-fold, which was not achieved by another transcription factor of the VvMYB family (*VvMYBA* or A2, respectively) (Figure 7 B + C). In Figure 7 D it can be seen that *VvABC12* was induced about 1.5-fold by VvMYB15 and VvMYBA2 and ca. 2.5-fold by VvMYB14. *VvGLUC1* and *VvGLUC2* both were induced by VvMYB15, but not VvMYB14, MYBA or MYBA2 (Figure 7 E + F).

In addition to these measurements, *VvGT5* and *VvLAC* were also investigated concerning their induction by VvMYB15 by Dr. K. Machemer-Noonan. Unfortunately, the constructs were cloned from

an unidentifiable cultivar and the system used for the dual luciferase assay was changed from cell culture particle gun bombardment to a protoplast-based system. Both promoters showed an induction increase comparable to *VvGT4* (personal communication, data not shown).

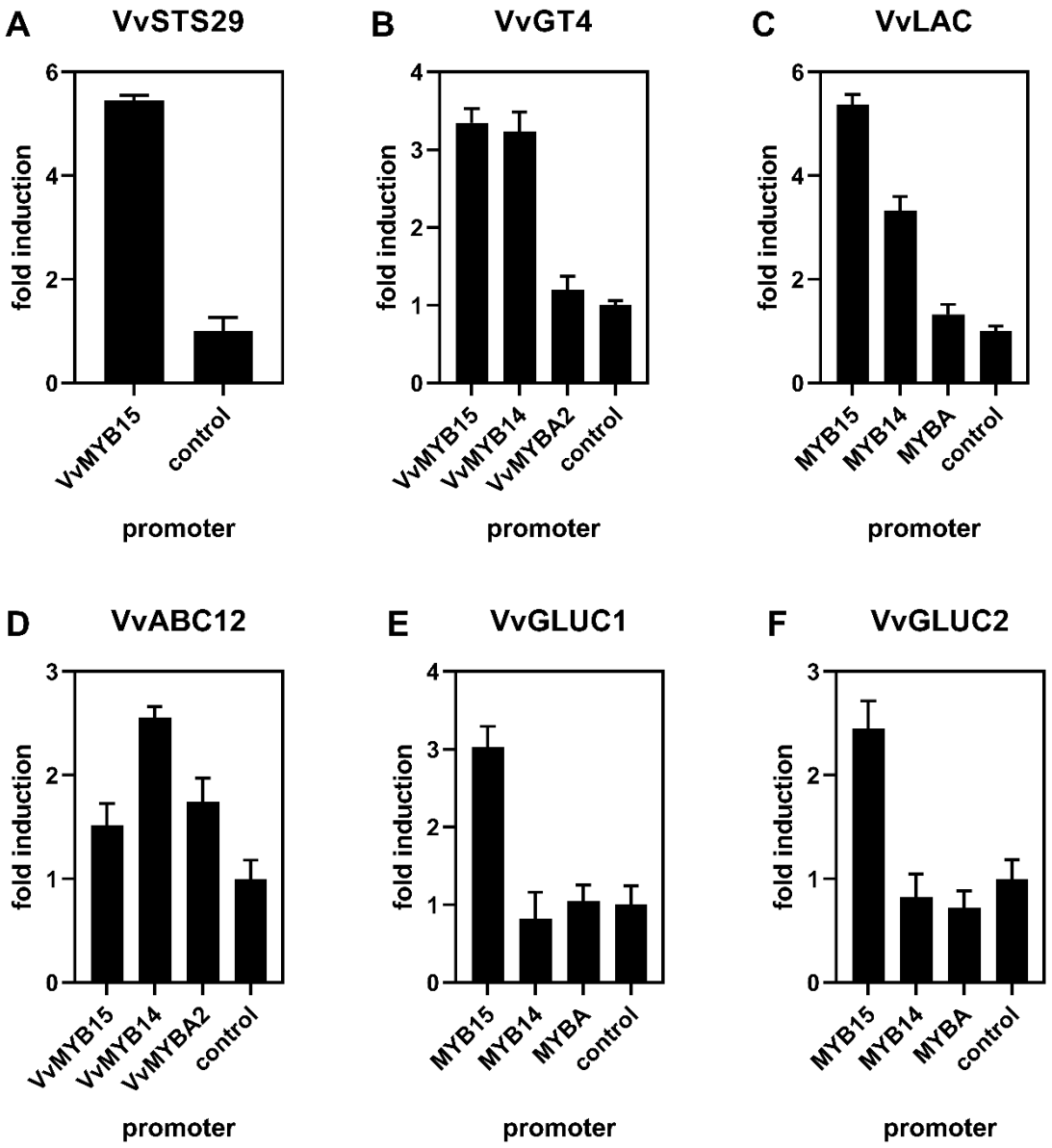


Figure 7: Induction of candidate gene promoters by VvMYB14 and VvMYB15 transcription factors measured via dual luciferase assay.

V.vinifera cv. 'Chardonnay' cell culture was used to perform a particle gun bombardment with subsequent dual luciferase promoter induction assays to determine the ability of transcription factors (VvMYB14, VvMYB15, VVMYBA, VvMYBA2 and empty control, indicated on the x-axis) to induce the promoters of *VvSTS29* (A), *VvGT4* (B), *VvLAC* (C), *VvABC12* (D), *VvGLUC1* (E) and *VvGLUC2* (F). TFs in pART7 vectors and promoters in pLUC vectors were co-transformed, substrate was added after 2 days of incubation and the luciferase signal was measured by photometer. Graphs show fold induction compared to the non-TF control, error bars indicate standard error, n=3 or 6.

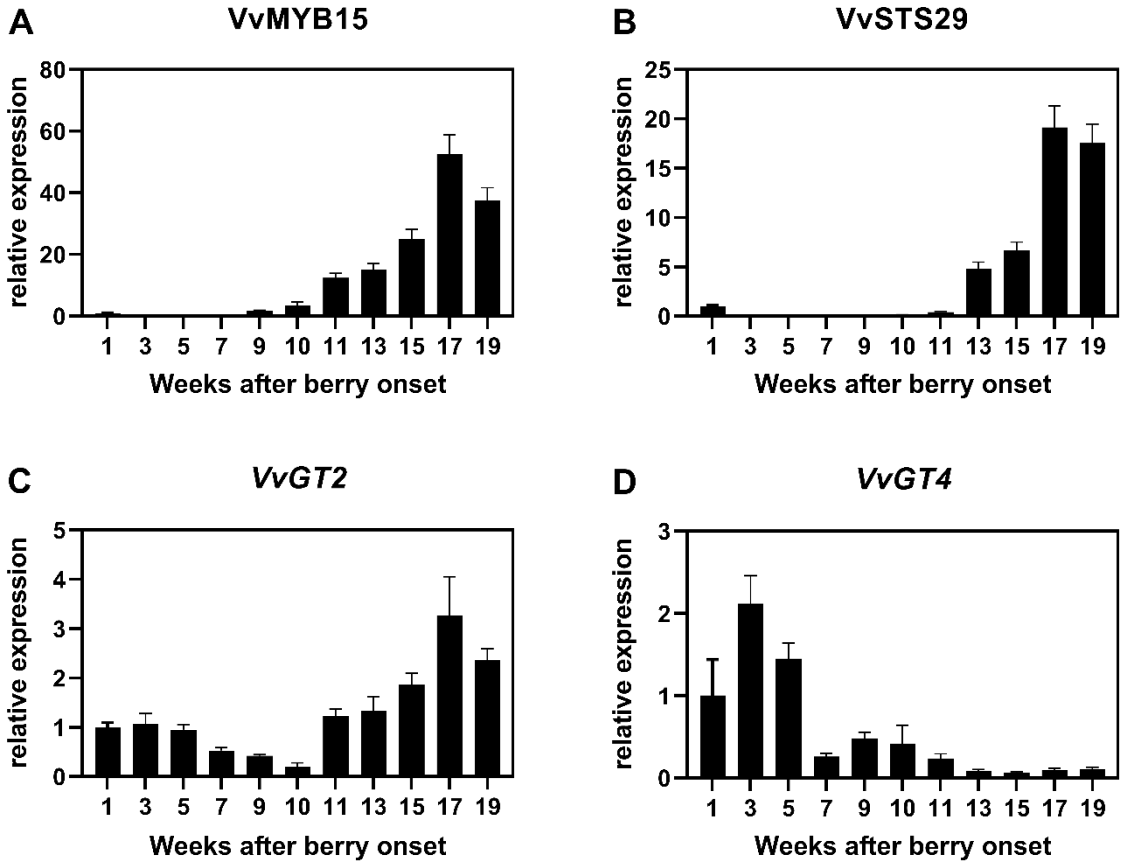
2.2 Correlation between gene expression and stilbene production in development and infection in *V. vinifera*

2.2.1 Glycosyltransferases *VvGT2* and *VvGT5* showed correlations between gene expression and stilbene content during grape berry ripening

The genes identified as potential candidates for stilbene-modifying enzymes or transporters were analyzed towards their gene expression in comparison with the stilbene-metabolite profiles. Metabolite profiles vary greatly during different developmental stages of grape berries (Kennedy, 2002; Conde et al., 2007). It is to be expected that the responsible genes show a similar trend as the products that are synthesized, modified or transported by the enzymes and transport-proteins encoded by these genes. Therefore, it was aimed to show a correlation between the gene expression of the TFs, STS and candidate genes with their respective (predicted) products, namely *trans*-resveratrol, *trans*-ε-viniferin (dimer) and *trans*-piceid (resveratrol-glycoside). In order to obtain a meaningful set of samples over the course of one season, 100 berries from different plants were collected every second week during the season with the exception of the time point at which growth switches to ripening (véraison) in the 10th week, when samples were taken weekly in order to closely monitor this important switch in expression and metabolite production. The berries were then analyzed for their transcript levels and stilbene content by qPCR and HPLC, respectively.

VvMYB15 and *VvSTS29*, the central transcription factor and the representative of the first dedicated stilbene enzymes in the pathway both showed an increase in their expression levels compared to the early weeks of berry development after vérasion with a maximum at week 17 of up to about 50-fold (VvMYB15) and about 20-fold (VvSTS29) (Figure 8 A + B). A similar pattern was recognizable in Figure 9 A, where *trans*-resveratrol accumulation increased strongly after véraison, from levels of below 100 ng per g freshweight in the berries to 500 – 1000 ng/g during véraison. A notable exception here was week one, which also showed a similarly high amount of *trans*-resveratrol. The two glycosyltransferases *VvGT2* and *VvGT5* (Figure 8 C + E) showed a comparable pattern to *VvMYB15* and *VvSTS29*, although a reduced relative expression in the late developmental stages of up to 3.5-fold (*VvGT2*) and 2-fold (*VvGT5*) relative to week one. *VvGT4* on the other hand did not correlate at all with this pattern of increasing expression after véraison, but rather reached its maximum in week two (Figure 8 D) and also showed low absolute expression levels (data not shown). The glycosyltransferases were expected to produce a glycosylated *trans*-resveratrol, of which *trans*-piceid is the most prominent and abundant. It followed the same pattern as *trans*-resveratrol, but with a much higher absolute level of below 1000 ng/g freshweight in the early lower samples and up to ca. 15000 ng/g

freshweight in the first week and weeks 17 and 19 (Figure 9 B). The last of the three analyzed stilbene compounds, *trans*-ε-viniferin, again showed a similar pattern as the two other analyzed stilbenes. *Trans*-ε-viniferin had a maximal accumulation of ca. 500 ng/g freshweight in week one, but did not reach this level again after véraison, staying at maximal 300 ng/g in week 19 (Figure 9 C). The laccase did not show a correlation to that, with the strongest relative expression in week one, but overall, almost negligible expression levels (Figure 8 F). The two ABC-transporters *VvABC12* and *VvABC13* showed expression levels that correlated well with *VvMYB15*, *VvSTS29* and the metabolites (Figure 8 G + H), although it cannot be said at this point whether they would have been expected to correlate with a specific stilbene or rather all of them, since they are thought to be responsible for stilbene transport. *VvROMT* would produce *trans*-pterostilbene, a methylated resveratrol, which was below the detection levels of our system in this experiment (Schmidlin et al., 2008). While the expression in the beginning of berry development did not correlate with the absent *trans*-pterostilbene, the later low expression levels did, showing that *VvROMT* and *trans*-pterostilbene were not constitutively expressed and produced in the analyzed developmental stages (Figure 8 I).



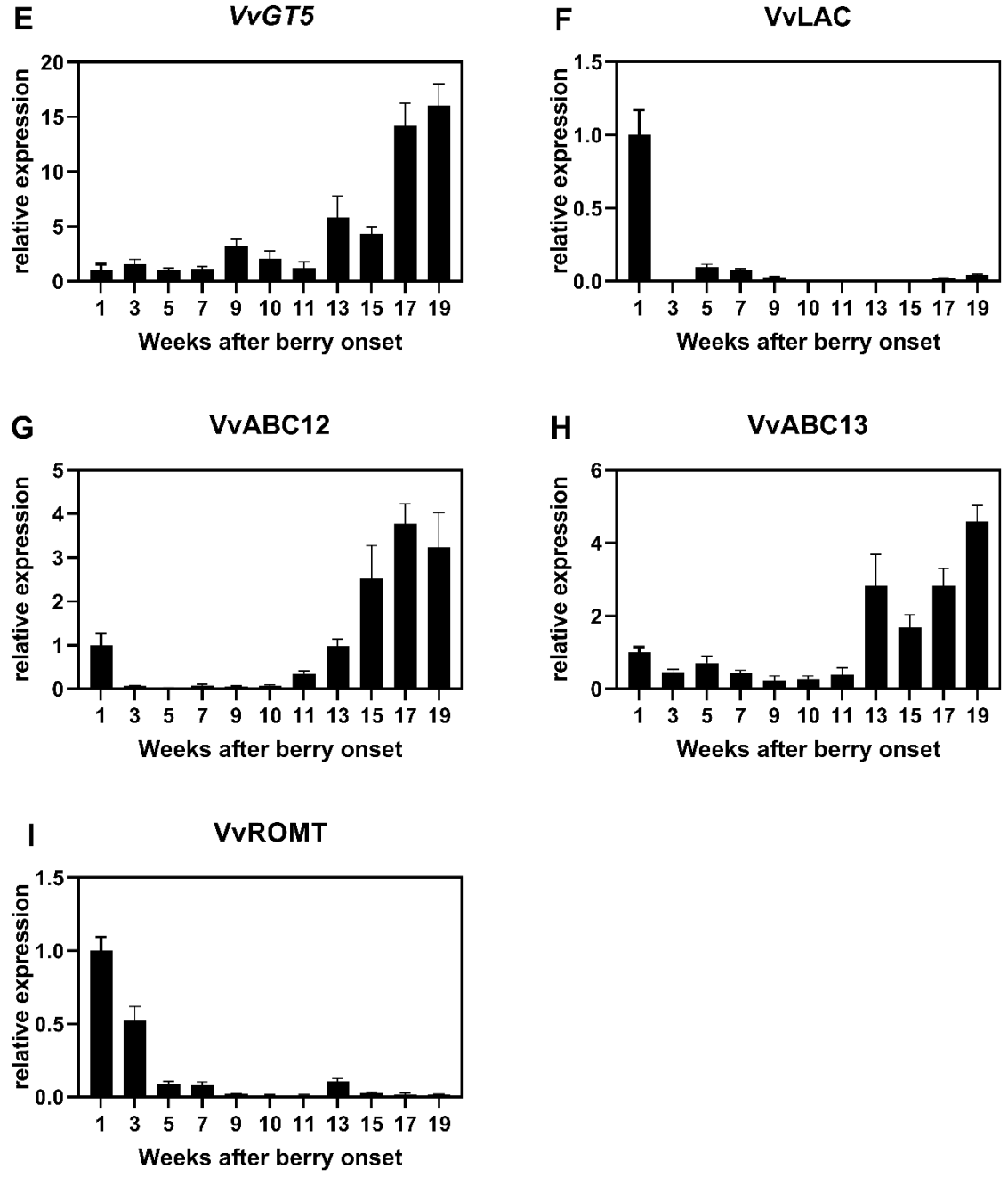


Figure 8: Expression levels of *VvMYB15*, VvSTS29 and selected candidate genes during berry ripening in *V. vinifera* cv. 'Pinot Noir' measured by qRT-PCR.

The expression of *VvMYB15* (A), *VvSTS29* (B), *VvGT2* (C), *VvGT4* (D), *VvGT5* (E), *VvLAC* (F), *VvABC12* (G), *VvABC13* (H) and VvROMT (I) in *V. vinifera* cv. 'Pinot Noir' grape berries during development from berry onset to the ripe state were determined by qPCR. 100 berries were collected per timepoint and pooled in the year 2011 in a vineyard near Schriesheim (Germany) every one to two weeks, as indicated by the time labels (1-19). The time point at which growth switches to ripening (véraison) was at week 10 in 'Pinot Noir'. The expression levels shown in each graph were normalized against *VvGADPH*, *VvEF1α* and *VvUBI*, expressed as mean values of one or two replicate PCRs (n=3 or n=6) and error bars indicating SEM. All expression levels are relative to their sample of week one, respectively.

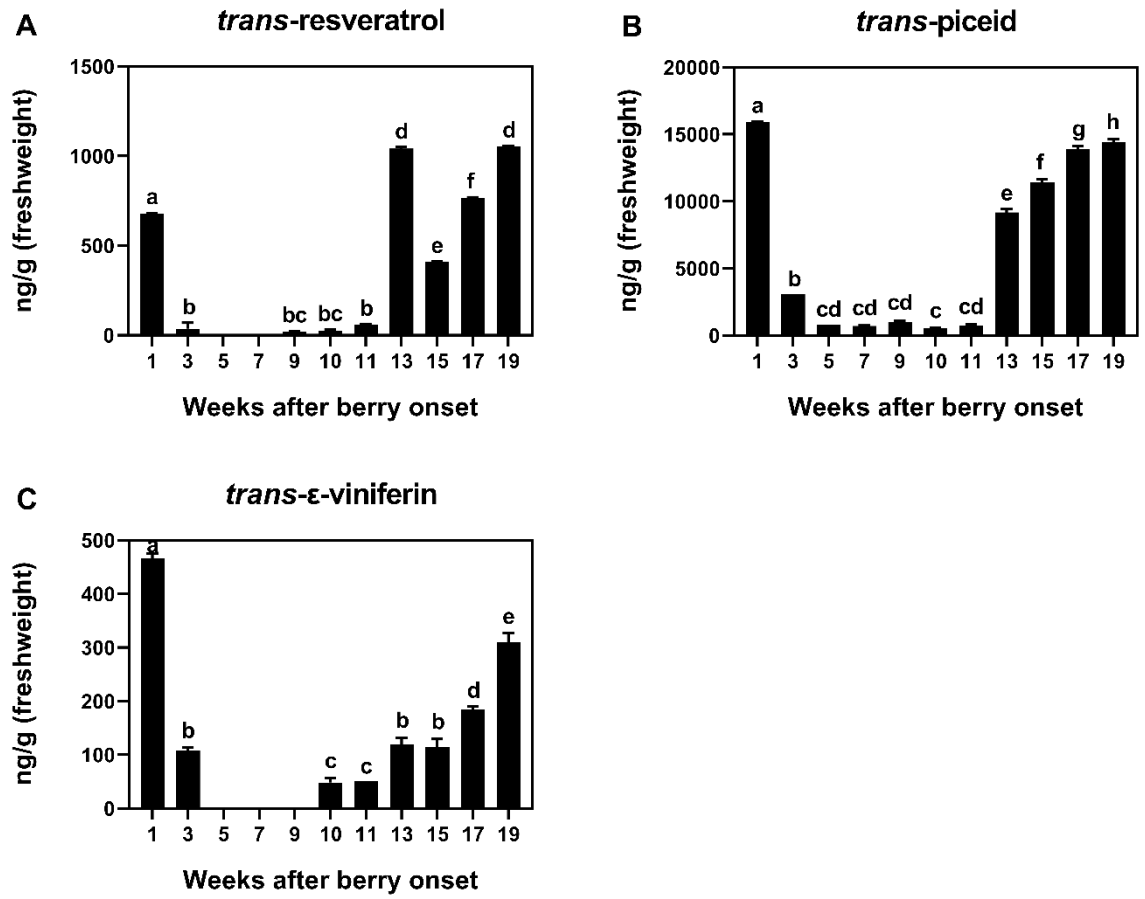
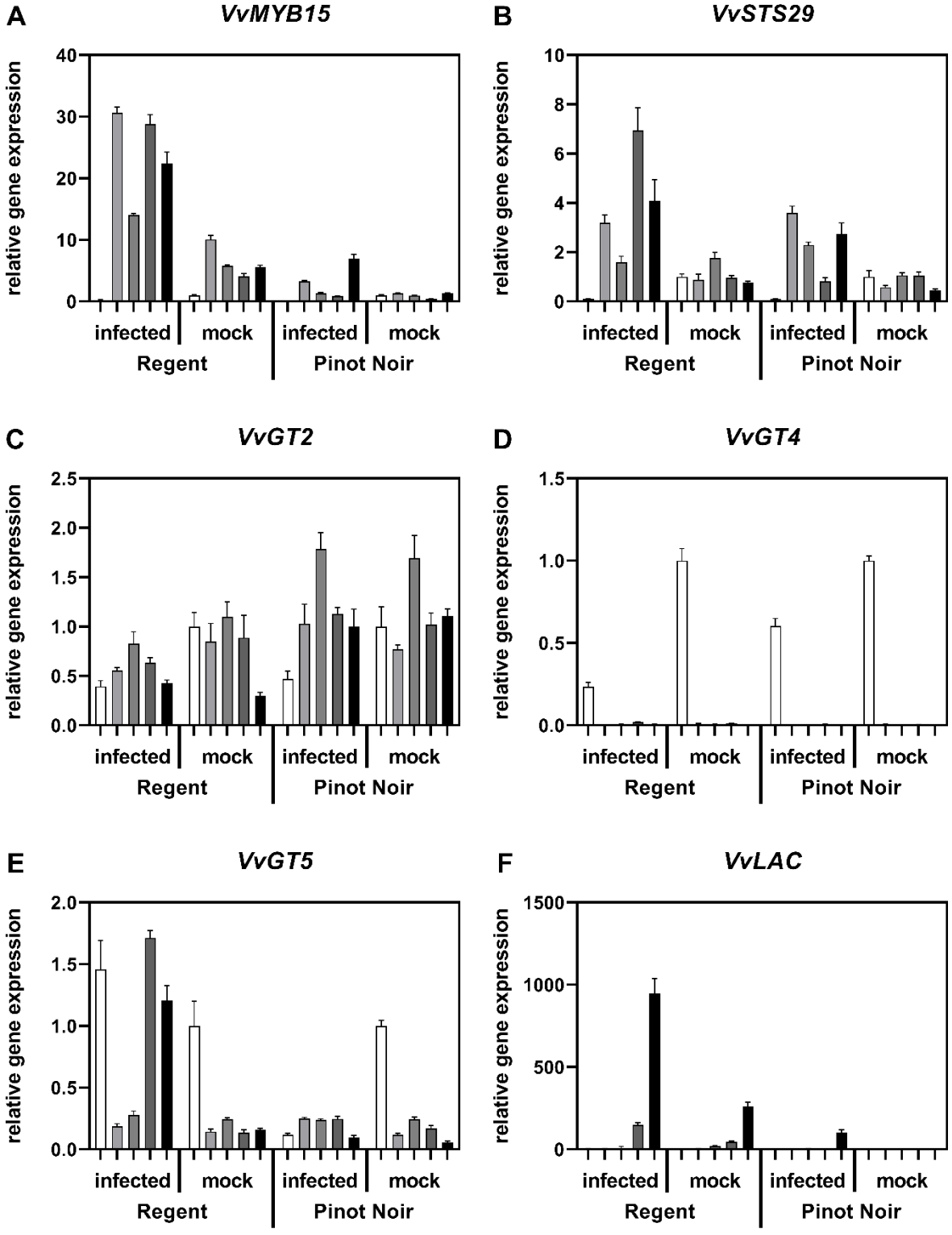


Figure 9: Amount of *trans*-resveratrol, *trans*-piceid and *trans*-*ε*-viniferin in berries of *V. vinifera* cv. 'Pinot Noir' during the time from berry onset to their ripe state.

The amount of *trans*-resveratrol (A), *trans*-piceid (B) and *trans*-*ε*-viniferin (C) during grape berry development from berry onset to the ripe state in *V. vinifera* cv. 'Pinot Noir' was determined by HPLC. The samples were a pool of 100 berries per timepoint collected 2011 in a vineyard near Schriesheim (Germany) every one to two weeks, as indicated by the time labels (1-19). The time point at which growth switches to ripening (véraison) was at week 10 in 'Pinot Noir'. The graphs show the amount of stilbenes in ng/mg (freshweight FW), expressed as mean values of three replicates (n=3) and error bars indicating standard deviation. An analysis of variance (ANOVA) was conducted to determine the effects of sampling time (weeks) on the content of *trans*-resveratrol, *trans*-piceid or *trans*-ε-viniferin content of the grape berries. The values were compared by Tukey's HSD test, different letters (a-h) indicate significant differences ($p < 0.05$).

2.2.2 Expression of *VvMYB15*, *VvSTS29*, *VvLAC* and *VvROMT* correlated with the accumulation of *trans*-resveratrol, *trans*-ε-viniferin and *trans*-pterostilbene after *P. viticola* infection

To get/gain further insight into the possible role of the candidate genes in stilbene production, transport or modification of stilbenes, their expression level and the amount of stilbenes during downy mildew infection was investigated. The expression profiles of the candidate genes were studied by qPCR in leaf discs infection assay. The grapevine cultivars 'Regent' and Pinot noir were inoculated with P. viticola or water. In contrast to the susceptible grapevine cultivar P. noir, Regent possesses a resistance locus (RPV3 locus) against P. viticola, mediating a moderate tolerance against the downy mildew pathogen. *VvMYB15* and *VvSTS29* (Figure 10 A + B) show a highly increased relative expression of up to 30-fold (*VvMYB15*) and up to 7-fold (*VvSTS29*) within the first 24 hpi in grapevine tissues of the resistant cultivar Regent compared to water controls (mock). The non-infected samples, as well as the infected 'Pinot Noir' sample, also showed an increase in expression of the two genes in the hours after infection, although only up to 10-fold (*VvMYB15*) and 4-fold (*VvSTS29*). These findings correlated with the measured *trans*-resveratrol levels, which increased from below 500 ng/g freshweight during the early infection phase (0 - 8 hpi) to 2000 ng/g after 24h in the resistant cultivar Regent, while the accumulation in the mock- and Pinot noir samples stayed below 500-1000 ng/g with one exception (Figure 11 A). Among the glycosyltransferases, there are no comparable trends (Figure 10 C - E). No increase of expression above 2-fold was observed in any of the samples and most samples showed a decreasing expression after *P. viticola* inoculation, especially for *VvGT4*. The *trans*-piceid accumulation on the other hand was higher compared to the *trans*-resveratrol level, starting at about 1000 ng/g freshweight and increased throughout all samples to 2000 ng/g or 3000 ng/g in the 48 h samples of 'Regent' infected and 'Pinot Noir' mock (Figure 11 B). T*rans*-*v*iniferin, one of the two active defense compounds besides *trans*-pterostilbene, was most abundant in the late, infected 'Regent' samples, although it could also be found in lower levels in the mock treated and 'Pinot Noir' samples (Figure 11 C). This approximately matched the laccase expression in 'Regent', although not in the 'Pinot Noir' mock treatment (Figure 10 F). The two ABC-transporters that were included in the study showed no trend towards increased expression during infection, but rather decreased in expression levels after infection (Figure 10 G + H), while the *VvROMT* (Figure 10 I) correlated well with the *trans*-pterostilbene content in 'Regent' during the defense response (Figure 11 D).


A
VvMYB15
relative gene expression
40
30
20
10
0
infected
mock
infected
mock
Regent
Pinot Noir
B
VvSTS29
relative gene expression
10
8
6
4
2
0
infected
mock
infected
mock
Regent
Pinot Noir
C
VvGT2
relative gene expression
2.5
2.0
1.5
1.0
0.5
0.0
infected
mock
infected
mock
Regent
Pinot Noir
D
VvGT4
relative gene expression
1.5
1.0
0.5
0.0
infected
mock
infected
mock
Regent
Pinot Noir
E
VvGT5
relative gene expression
2.0
1.5
1.0
0.5
0.0
infected
mock
infected
mock
Regent
Pinot Noir
F
VvLAC
relative gene expression
1500
1000
500
0
infected
mock
infected
mock
Regent
Pinot Noir

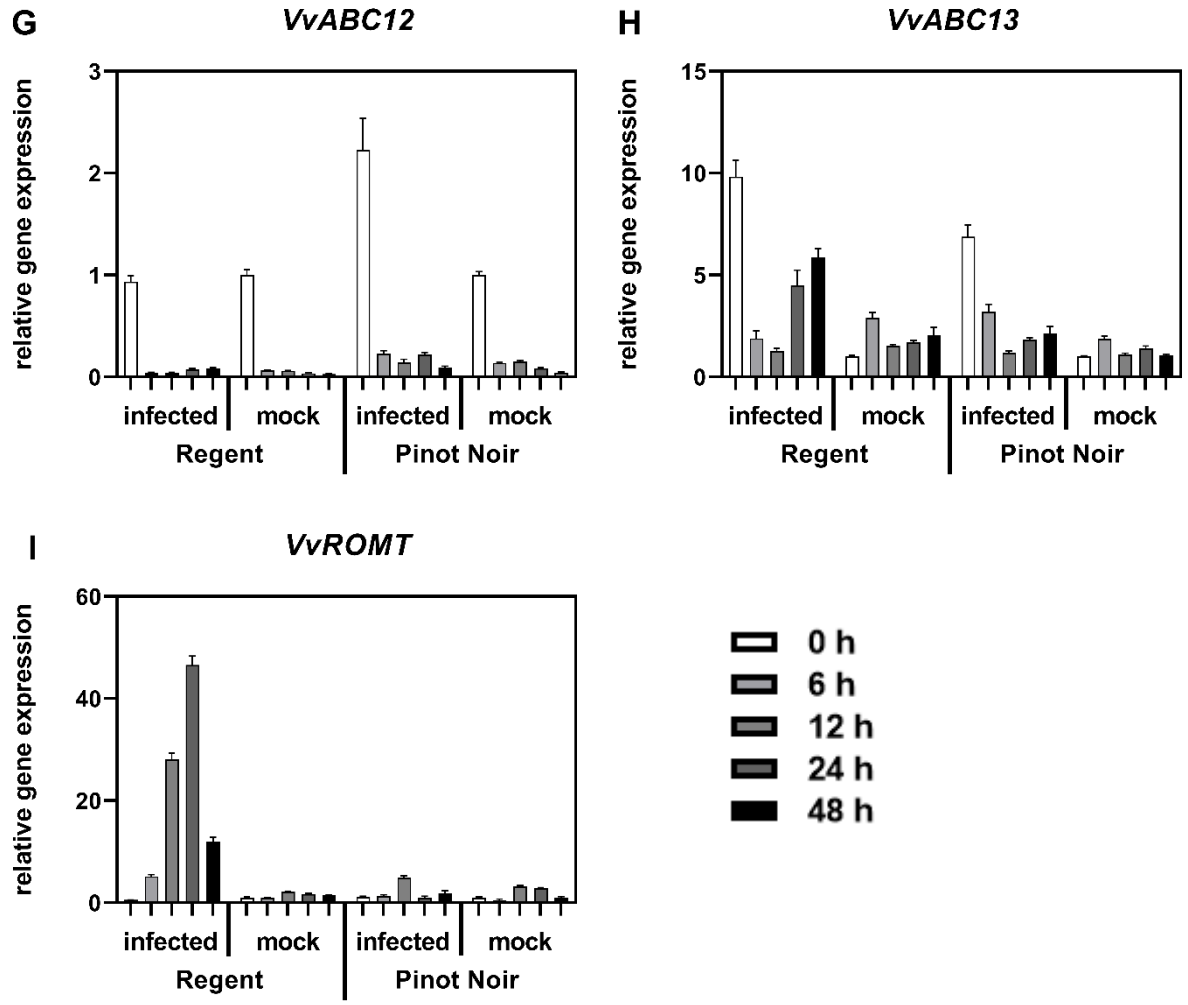


Figure 10: Expression of candidate genes during *P. viticola* infection of *V. vinifera* leaf discs of the 'Regent' and 'Pinot Noir' cultivar.

Gene expression levels of *VvMYB15* (A), *VvSTS29* (B), *VvGT2* (C), *VvGT4* (D), *VvGT5* (E), *VvLAC* (F), *VvABC12* (G), *VvABC13* (H) and *VvROMT* (I) in *V. vinifera* cv. 'Regent' and 'Pinot Noir' leaf discs infected with *Plasmopara viticola* or mock treated were determined by qRT-PCR. The leaf discs were sampled 0 h, 6 h, 12 h, 24 h and 48 h after infection or water treatment (mock), as displayed in the figure legend. The expression levels shown in each graph were normalized against *VvGADPH*, *VvEF1α* and *VvUBI*, expressed as mean values of one or two replicate PCRs (n=3 or n=6) and error bars indicating SEM. All expression levels displayed are relative to their 0 h mock sample, respectively.

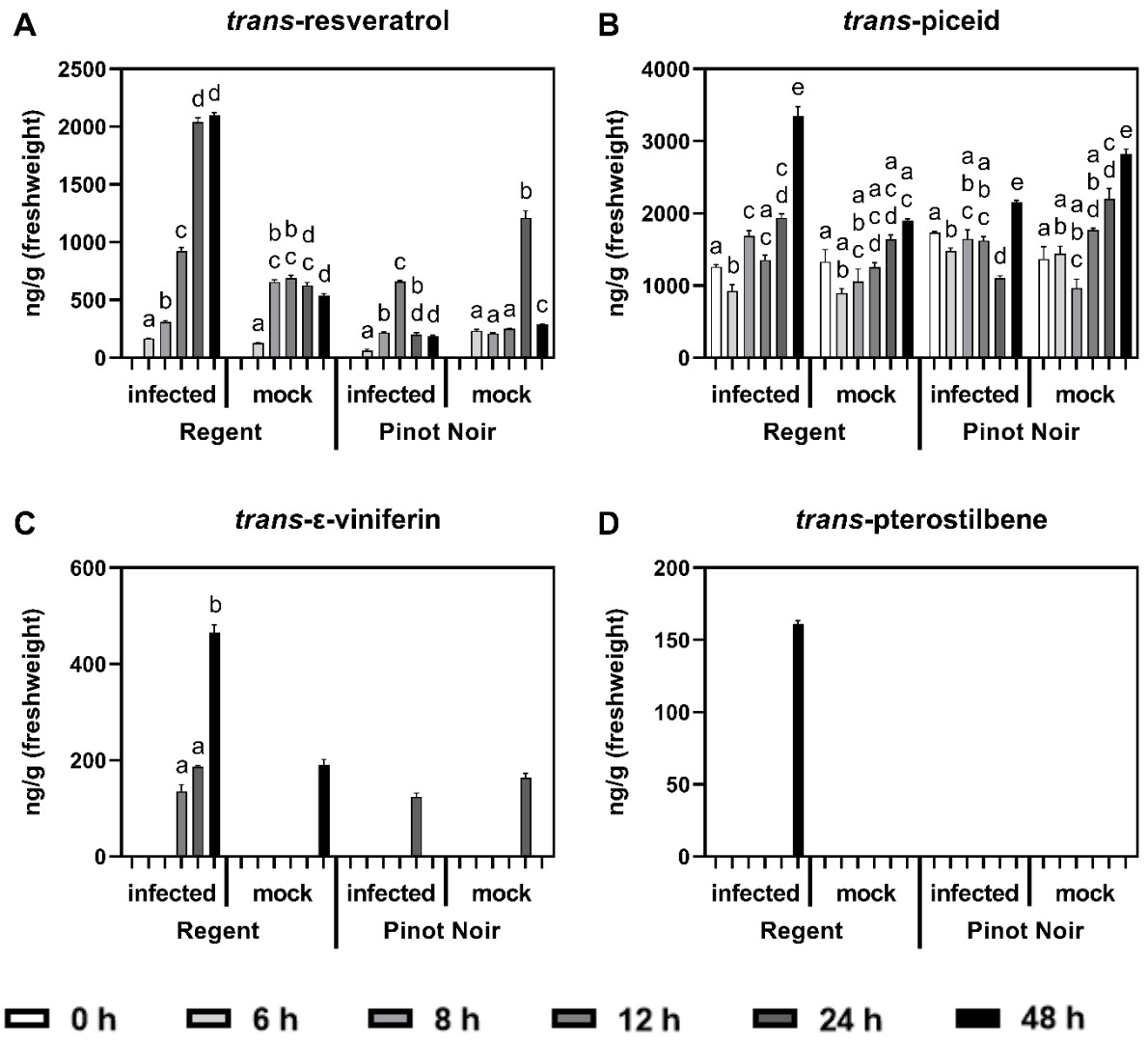


Figure 11: Amount of selected stilbenes in *V. vinifera* leaf discs of the 'Regent' and 'Pinot Noir' varieties during *P. viticola* infection.

The amount of *trans*-resveratrol (A), *trans*-piceid (B), *trans*-ε-viniferin (C) and *trans*-pterostilbene (D) in *V. vinifera* cv. 'Regent' and 'Pinot Noir' leaf discs infected with *Plasmopara viticola* or mock treated was determined by HPLC. The leaf discs were sampled 0 h, 6 h, 8 h, 12 h, 24 h and 48 h after infection or water treatment (mock) (see figure legend). The graphs show the amount of stilbenes in ng/g (freshweight), expressed as mean values of three replicates (n=3) and error bars indicating standard deviation. An analysis of variance (ANOVA) was conducted to determine the effects of sampling time (hpi) on the content of *trans*-resveratrol, *trans*-piceid, *trans*-ε-viniferin or *trans*-pterostilbene content of the grape berries. The values were compared by Tukey's HSD test, different letters (a-e) indicate significant differences between timepoints within one cultuvar+treatment column ($p < 0.05$). For statistical comparison between the cultivars and treatments within one sampling time group, see Supplemental Figure 1.

2.2.3 Comparison of berry onset and ripe berry stage in different cultivars revealed no clear correlation between candidate gene expression and stilbene content

Beside the fungus resistant cultivar 'Regent', that possesses the Rpv3 resistance locus, other resistant cultivars gain more and more importance in viticulture. Beside the Rpv3- locus two other main loci were used in grapevine breeding, the Rpv10 and Rpv12 loci. The differences in candidate gene expression and stilbene accumulation between the Rpv3- cultivar 'Regent' and the susceptible cultivar 'Pinot Noir' during *P. viticola* infection was already shown. It is known that the different Rpv-loci mediate different levels of resistance towards *P. viticola* (Eisenmann et al., 2019). To gain insight into the role of stilbenes during berry development in fungus resistant cultivars and to gain more data on the correlation between stilbene content and gene expression, five additional cultivars were investigated. The inflorescences of 'Muscaris' (Rpv 10), 'Calardis Blanc' (Rpv3.1 + 3.2), 'Cabernet Blanc' (Rpv 3.1), 'Cal6-04' (Rpv 3.1 + 12) and 'Riesling' (no Rpvs), as well as their ripe berries in fall were collected and analyzed via qPCR and HPLC.

The gene expression of *VvMYB15*, *VvSTS29* and the selected candidate genes relative to the 'Riesling' sample varied strongly not only between the cultivars but also between the genes (Figure 12). An interesting comparison would have been between 'Cabernet Blanc' and 'Riesling', since they possess the same combination of resistance loci as in the infection series experiment: 'Regent' and 'Cabernet Blanc' with the Rpv 3.1 and 'Pinot Noir' and 'Riesling' with no Rpv locus. The only cases where the expression was increased in 'Cabernet Blanc' were *VvMYB15* (1,8- fold), *VvGT2* (3,5-fold) and *VvROMT* (3-fold) (Figure 12 A, C, I). These values were much lower than in the infection experiment. In general, there was a high variance between the genes and cultivars with no clear trend of consistently higher gene expression in a specific cultivar. The stilbene levels (Figure 13) reflected this with the high background levels of *trans*-piceid (10000 ng/g freshweight in flowers and 100-1000 ng/g freshweight in ripe berries and very low levels of *trans*-resveratrol and *trans*-*ε*-viniferin in the same tissues.

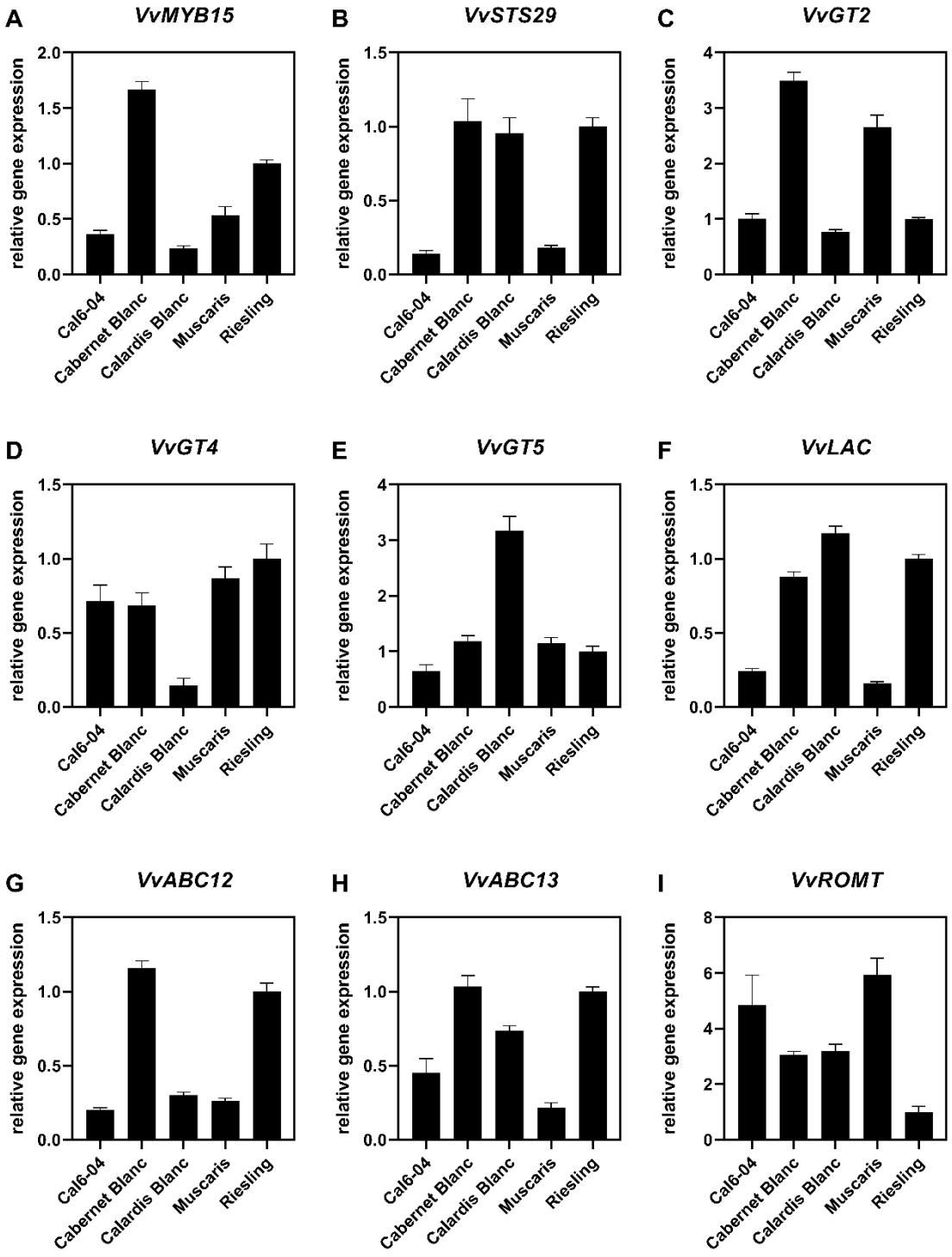


Figure 12: Gene expression levels of *VvMYB15*, *VvSTS29* and selected candidate genes in flowers of different *V. vinifera* cultivars, measured by qPCR.

Expression levels of *VvMYB15* (A), *VvSTS29* (B), *VvGT2* (C), *VvGT4* (D), *VvGT5* (E), *VvLAC* (F), *VvABC12* (G), *VvABC13* (H) and *VvROMT* (I) in *V. vinifera* flowers (just before berry formation) of the cultivars 'Cal6-04', 'Cabernet Blanc', 'Calardis Blanc', 'Muscaris' and 'Riesling'. Samples included tissue material of 5 different plants, analysis was done by qPCR. The expression levels shown in each graph were normalized against *VvGADPH*, *VvEF1α* and *VvUBI*, expressed as mean values of one or two replicate PCRs (n=3 or n=6) and error bars indicating SEM. All expression levels are relative to the 'Riesling' sample as only non-resistant variety.

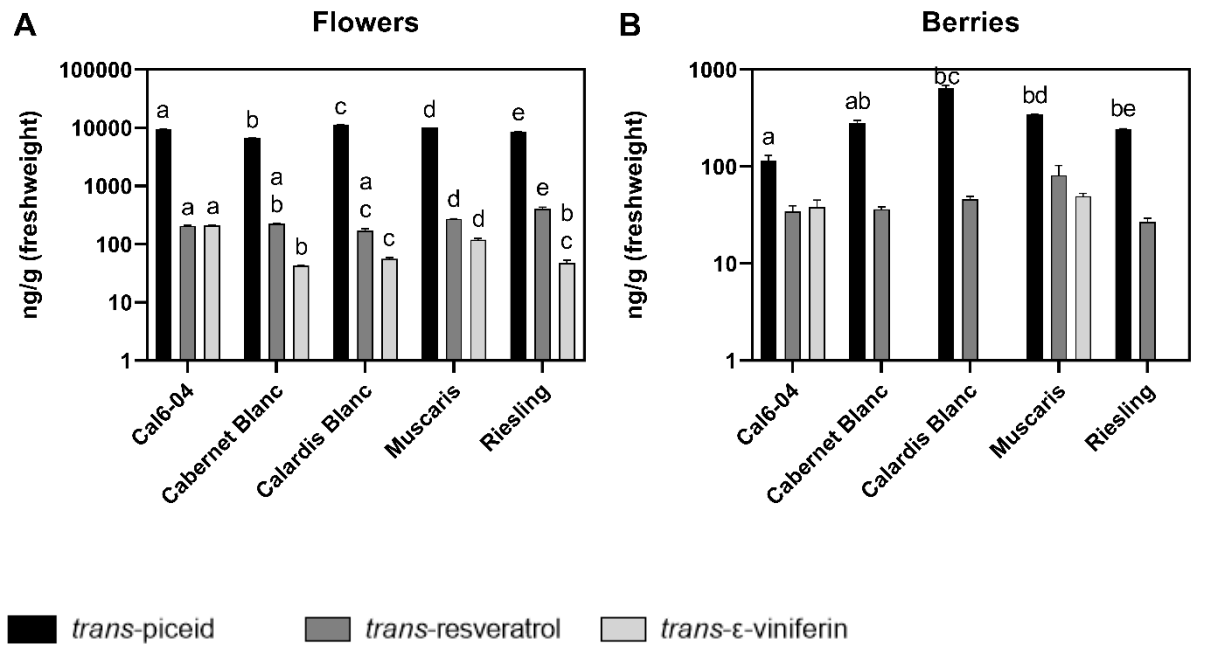


Figure 13: Accumulation of *trans*-resveratrol, *trans*-piceid and *trans-ε*-viniferin in flowers and berries of different *V. vinifera* varieties, measured by HPLC.

Levels of *trans*-resveratrol, *trans*-piceid and *trans*-ε-viniferin in *V. vinifera* flowers (just before berry formation; A) and ripe berries (B) of the varieties 'Cal6-04', 'Cabernet Blanc, 'Calardis Blanc', 'Muscaris' and 'Riesling'. The plants were grown in the vineyards at the DLR (Neustadt an der Weinstraße, 2018), samples include tissue material of 5 different plants. The graphs show the amount of stilbenes in ng/g (freshweight), expressed as mean values of three replicates (n=3) and error bars indicating standard deviation. The Y-axis is on $\log_{10}$ scale. An analysis of variance (ANOVA) was conducted to determine the effect of cultivar on the content of *trans*-resveratrol, *trans*-piceid or *trans*-ε-viniferin content of the flowers and a mixed effects analysis was conducted on the berry material. The values were compared by Tukey's HSD test, different letters (a-e) indicate significant differences between cultivars ($p < 0.05$).

2.3 Protein expression in different systems, enzyme extraction and biochemical characterization of candidate genes

2.3.1 Low levels of trans-piceid in resveratrol-producing *S. cerevisiae* cultures transformed with *VvGT2*

Studies that place the identified candidate genes in the stilbene biosynthesis- and modification pathway by promoter induction assay or correlation study are essential tools in narrowing down the number of promising candidate genes, but they cannot replace further studies which investigate the biochemical processes involved in the synthesis of modified stilbenes more directly. A popular system to achieve this are yeast expression systems. The goal of this experiment was to use *S. cerevisiae* strains expressing grapevine genes for *trans*-resveratrol synthesis and transport, provided by the cooperation partner Evolva, transform them with the most promising candidate genes and investigate, by HPLC, whether or not the expected stilbene products (or other stilbenes) are produced from the *trans*-resveratrol provided by the cells.

While the *trans*-resveratrol content reached its end values of 1,000 – 3,500 ng/ml medium, depending on the culture (with the VvGT4 control and VvLAC induced cultures failing to show any comparable *trans*-resveratrol levels), *trans*-piceid was the only other stilbene that could be found (Figure 14). It was present in the 24 h VvGT2 (induced) sample at 46 ng/ml of medium. *Trans*-ε-viniferin was not detected in the laccase containing culture. The levels of *trans*-resveratrol compared between the sampled cultures roughly correlates with their optical density (Supplemental Figure 2).

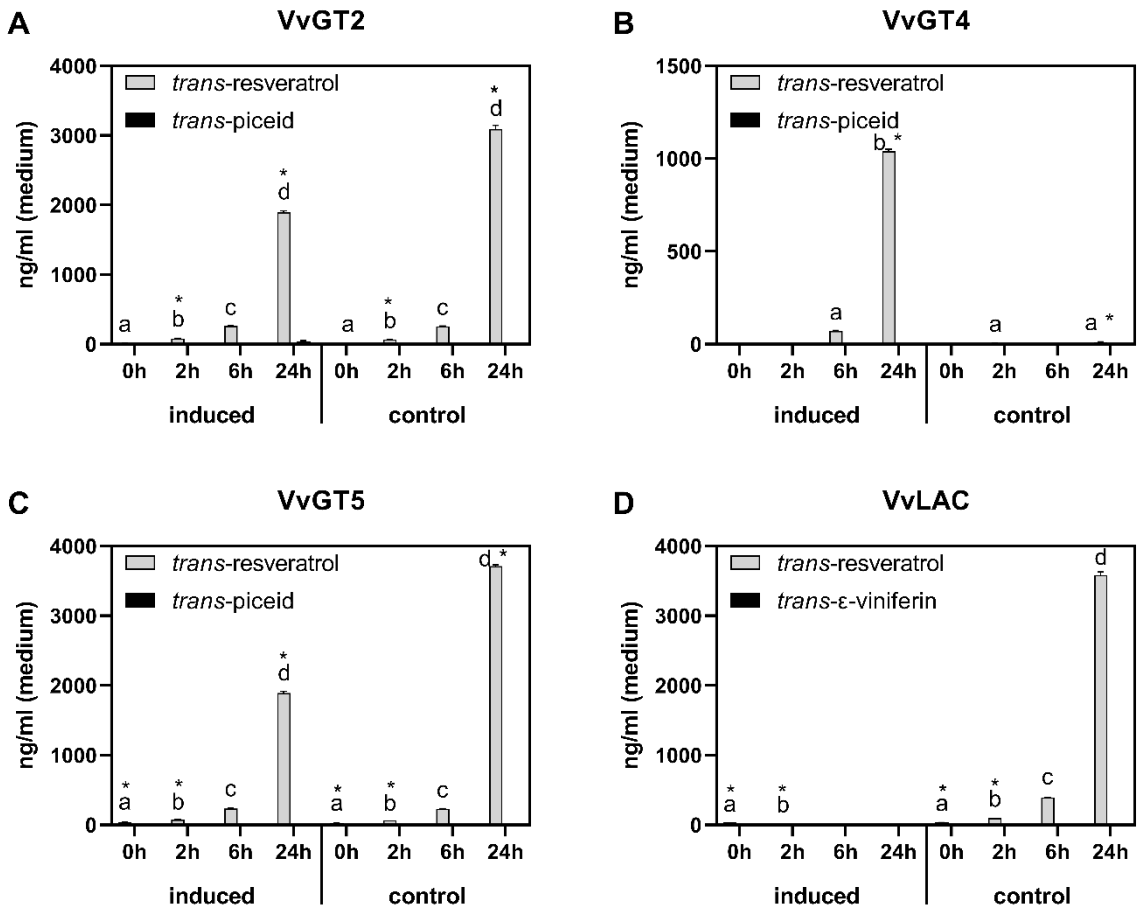


Figure 14: *Trans*-resveratrol, *trans*-piceid and *trans-ε*-viniferin produced in genetically modified *Saccharomyces cerevisiae* after growth of up to 24 h measured by HPLC.

S. cerevisiae strains expressing grapevine genes for resveratrol synthesis and transport, provided by the cooperation partner Evolva were transformed with the candidate genes *VvGT2* (A), *VvGT4* (B), *VvGT5* (C) and *VvLAC* (D). The cells were grown in induction medium or non-inducing control medium, the stilbenes were extracted and measured by HPLC. The stilbene content is given in ng/ml medium including cells, taken after 0 h, 2 h, 6 h or 24 h. Error bars indicate standard deviation. An analysis of variance (ANOVA) was conducted to determine the effects of sampling time (hours) and treatment (induced vs. control) on the content of *trans*-resveratrol, *trans*-piceid or *trans*-ε-viniferin of the medium. The values were compared by Tukey's HSD test, different letters (a-d) indicate significant differences between timepoints within one treatment column and asterisks indicate significant difference between the same sampling timepoint within one treatment ($p < 0.05$).

2.3.2 VvGT2 and At73B5 were able to metabolize *trans*-resveratrol into *trans*-piceid, although with less efficiency than they can other substrates

In a second step towards characterization of genes involved in stilbene biosynthesis and modification, the enzymes were investigated for their substrate specificity. This was done in cooperation with Dr. Philippe Hugueney and his group "Métabolisme Secondaire de la Vigne" at the INRAE Colmar, France. The candidate genes (*VvGT2*, *VvGT4*, *VvGT5* and *VvLAC*) were cloned into bacterial expression vectors (pHNGWA) with NusA tags for better solubility (after pre-testing determined a very low solubility of the enzymes under non-denaturing conditions, data not shown). The constructs were transformed into BL21 DE3 cells and used in an enzymatic assay with a wide variety of possible substrates including resveratrol, flavonoids (kaempferol, quercetin) or terpenoids (geraniol, linalool). The assays included the *Arabidopsis thaliana* glucosyltransferase *UGT73B5*, which is used as an unspecific control (positive control), *VvGT2* and *VvGT5* as well as two controls, one being untransformed BL21 DE3 cells and one being the assay without cell extract. Cloning of *VvGT4* did not succeed in time for this project.

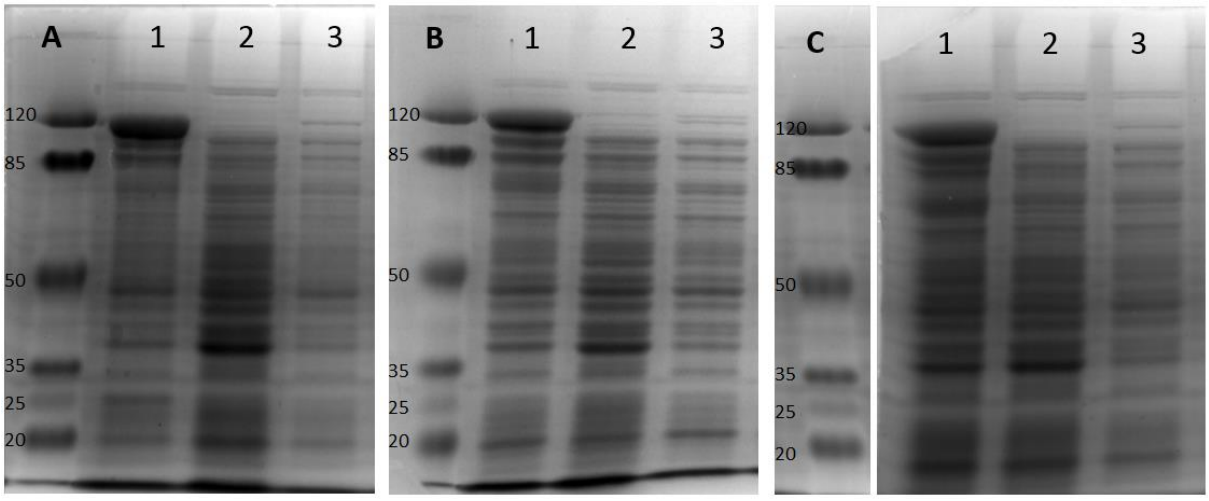


Figure 15: Protein extract of *E.coli* strains expressing VvGT2, VvGT5 or an *A.thaliana* glycosyltransferase (*At73B5*).

An *Arabidopsis thaliana* glycosyltransferase (*At73B5*) (A), *VvGT2* (B) and *VvGT5* (C) were cloned into pHGGWA vectors (NusA-tag for better solubility) and transformed into BL21 DE3 *E.coli* strains. SDS-PAGE of the complete protein extracts were prepared. Lane one shows the crude extract of the induced culture (1 mM IPTG, 4 h), lane two the same culture uninduced and lane three the protein content in the supernatant of the induced culture (lane one). The leftmost lanes are marker lanes, molecular weight in kDa (Pierce™ prestained protein molecular weight marker). Figure C was cut due to sample arrangement on the PAA gel with other samples, for uncut picture see Supplemental Figure 3.

The constructs were induced with 1 mM IPTG. The products were analyzed via UHPLC-MS. As depicted in Figure 15, *At73B5*, *VvGT2* and *VvGT5* were successfully expressed and present in a crude extract of the cells. While the majority remained insoluble and was discarded in the pellet, a sufficient amount could be preserved in the supernatant. *VvLAC* was cloned into the system, but no expression was observed, despite several attempts with other strains or methods, e.g. arctic express strains or modifications of the medium. The results with the three main substrates (resveratrol, kaempferol and quercetin) are displayed in Figure 16. The graphs depict one experiment as representative of 3 independent experiments with a fresh batch of the same stain, which resulted in similar trends but varying raw signal. The signal strength varied between the different substances and thus cannot be directly compared but only taken as an indication for the presence or absence as compared to the others. The *Arabidopsis* GT 73B5 was able to accept and glucosylate the three substrates resveratrol, kaempferol and quercetin, which is evident by the decrease in available substrate and the presence of peaks in the chromatograms with the distinct masses of the respective glycosylated substrates (indicated by the "G" above the columns in Figure 16). *VvGT2* was able to use esculetin and the flavonoids as substrates, but also to a lower extend *trans*-resveratrol as shown before in the publication by (Hall and De Luca, 2007). *VvGT5* showed even lower product levels and no apparent usage of *trans*-resveratrol (Figure 16 A).

Since the efficiency of those three substrates mediated by VvGT2 and VvGT5 was low or inefficiently, more substrates were tested in the same assay in order to find the original substrate of these enzymes. A total of 17 polyphenols and 11 further common substrates in a 10 µM concentration as well as UDP-glucose (1 mM) were used in these assays. Of these, caffeic acid, ethyl-gallate, naringenin and myricetin showed glucosylation patterns Table 4.

Table 4: Additional potential substrates for the investigated glycosyltransferases as discovered in an enzymatic assay with a substrate mix, measured by UHPLC-MS.

The glycosyltransferases At73B5, VvGT2 and VvGT5 transformed into BL21 DE3 E.coli strains (BL21 DE3), which then were provided with a substrate mix containing 17 polyphenols and 11 further common substrates in a 10 µM concentration as well as UDP-glucose (1 mM). Besides the previously investigated substrates (Figure 16), caffeic acid, ethyl-gallate, naringenin and myricetin were also glucosylated. See Supplemental Figure 5 for myricetin chromatograms as example for the analysis.

Substrate	Glycosylated by
Caffeic acid	VvGT2
Ethyl gallate	73B5, VvGT2
Naringenin	73B5, VvGT2, VvGT5 (less by BL21 +ctrl)
Myricetin (example graphs)	73B5, VvGT2, VvGT5

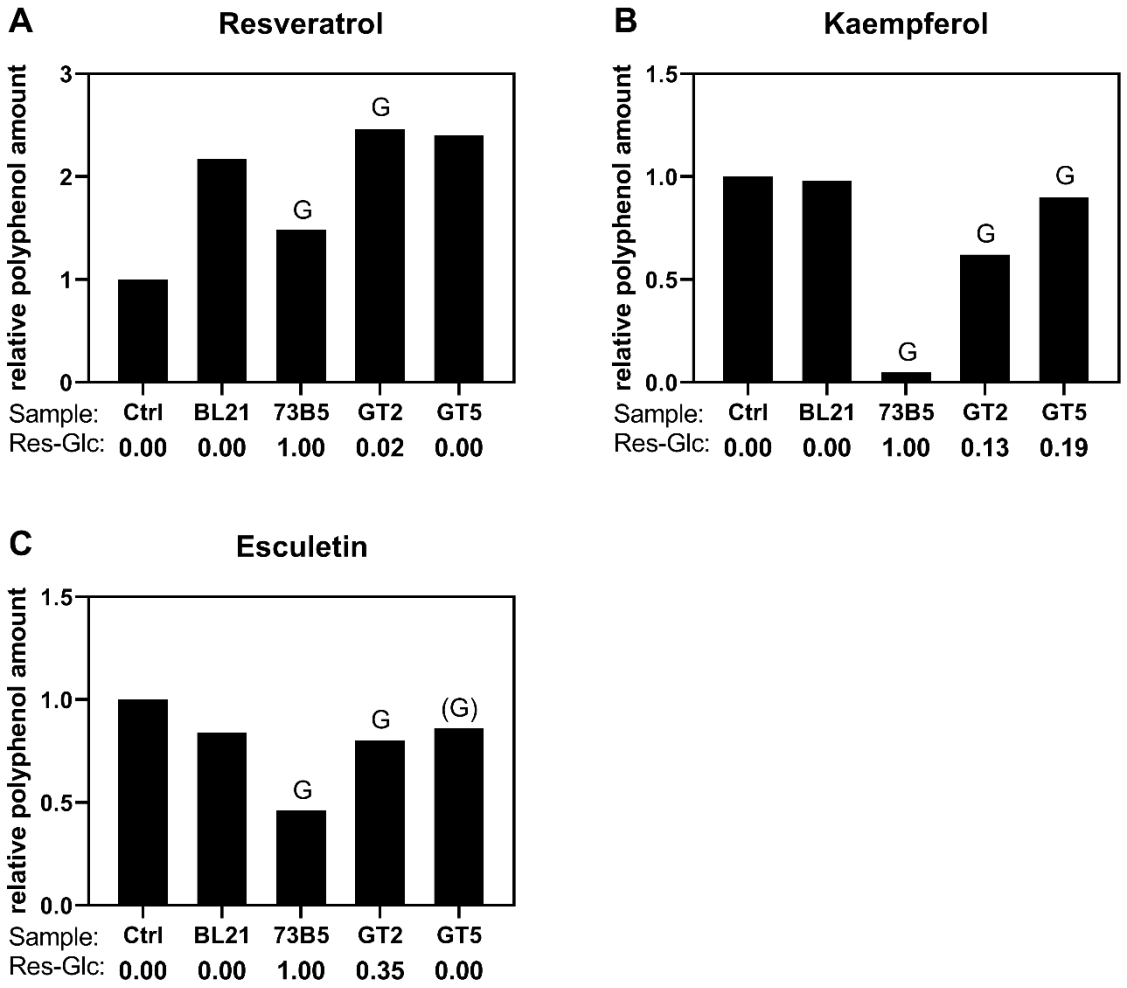


Figure 16: Relative amount of selected polyphenols measured by UHPLC-MS after protein extraction and enzymatic assay.

The glycosyltransferases *At73B5*, *VvGT2* and *VvGT5* were transformed into an BL21 DE3 *E.coli* strain, which then were provided with the substrates *trans*-resveratrol (A), Kaempferol (B) and Esculetin (C) in a 10µM concentration as well as UDP-glucose (1mM) for an overnight enzymatic assay. The amount of remaining substrate was measured by UHPLC-MS and is displayed as peak area relative to the control sample. Findings of glycosylated substrate at the same specific mass (distinguished by retention time) are indicated by a "G" above the bar. The line below the x-axis description indicates this amount of glycosylated substrate relative to the amount found in At73B5 (Esculetin GT5 shows a peak there, but it is too small to be calculated and represented in this way). Due to the missing quantification, peak areas of the glycosylated mass cannot be compared and are not depicted. The graphs depict one experiment, which are representative of 3 independent experiments with a fresh batch of the same stain, which resulted in similar trends but varying raw signal. See Supplemental Figure 4 for example and explanation of the resveratrol raw data.

2.3.3 *VvLAC* expression in *N. benthamiana* slightly increases the amount of an unknown substance with very similar retention properties as *trans*-ε-viniferin

For the investigation of potential products of the laccase, a plant expression system was utilized in order to circumvent the troubles with the previously described bacterial system. For this purpose, *VvSTS29* and *VvLAC* were expressed in tobacco plants in different combinations and the extract of these leaves were used for an enzymatic assay. After allowing the plant to express enzymes for 48 h to 96 h, the leaf was homogenized and the resulting extract incubated with *trans*-resveratrol in an enzymatic assay, however, no *trans*-ε-viniferin production was observed in HPLC measurement.

While no *trans*-ε-viniferin could be found in the samples, a substance eluting within 3-4 seconds of the *trans*-ε-viniferin standard was found (Supplemental Figure 6). This substance was present only in the 72 h and 96 h samples, but in all enzyme combinations (one sample of day two did not show any, but might be due to measurement error). The amount increases slightly only in the samples that have both enzymes.

3 Discussion

3.1 Microarray-, in silico-, and promoter induction analysis enabled identification and filtering of candidate genes

The goal of this project was to identify target genes of the VvMYB14 and VvMYB15 transcription factors and thus revealing genes involved in the biosynthesis, modification or transport of stilbenes in *Vitis vinifera*. These two transcription factors were identified as main regulators of the stilbene biosynthesis pathway in grapevine by Höll et al. (2013; 2014).

To this end, they conducted microarray experiments with different *V. vinifera* tissues with increased *VvMYB15* expression. With these datasets available at the start of the project, a large number of potential candidate genes that showed elevated expression levels in *VvMYB15* overexpressing samples were identified. In this first part of the project, the upregulation of the candidate genes was confirmed via qRT-PCR analysis of the same tissues. Furthermore, *in silico* analysis were conducted, in order to get some initial ideas about location and/or function of the enzymes and transporters identified by this approach. Additionally, promoter induction assays of the candidate genes were performed, to confirm their placement in the regulation network downstream of the two MYB TFs.

3.1.1 Approach of overexpressing *VvMYB15* for microarray revealed promising candidate genes and holds even more potential for future findings

Two DNA microarray (MA) measurements were performed with samples from three different experiments. Microarray A was done with *V. vinifera* cv. 'SO4' leaves from plants ectopically expressing *VvMYB15* as well as tissue from *V. vinifera* cv. 'Chardonnay' hairy root culture ectopically expressing *VvMYB15*. Microarray B was performed with leaves from *V. vinifera* cv. 'Shiraz' that were transformed with pKGWFS7:VvMYB15 or an empty control vector via agrobacterium mediated infiltration. The candidate genes found in either array A or array B differ between the two datasets (Table 2). This is likely explainable by the nature of the datasets. While array A has 30,000 probesets, covering about 16,000 genes (which corresponds to roughly half of the predicted grapevine genes (Jaillon et al., 2007; Grape Genome Browser, 2020)), there are only 16,000 probes in array B, which could explain some of the missing genes that were found in array A, but not array B. Furthermore, only the genes that showed a fold change of 2 or more were included in the analysis. This threshold was chosen in order to filter out most of the random changes between the *VvMYB15* overexpressing plants and their controls. Also,

the downregulated genes were not analyzed in this project, since they might indeed be targets of the transcription factor, but are not part of the stilbene biosynthesis pathway, if they are switched off by the transcription factor responsible for stilbene production. This leaves us with just over 300 genes with 2-fold increased expression in microarray A, and just over 200 in microarray B (data not shown). The genes left in the analysis include, besides many yet uncharacterized genes, other transcription factors (e.g. WRKY and related proteins), stilbene synthases (11 in MA-A and 10 in MA-B), transport proteins and modifying enzymes like transferases for a variety of substrates and transferred molecules. The further selection of the enzymes was done in reverse by looking at potentially interesting stilbene functions in grapevine. Glycosylated stilbenes and in grapevine specifically piceid are potential storage forms or might be working in detoxification and inactivation towards a role as phytoalexins and therefore might be interesting with regard to the amount of stilbenes present in cells at certain times and their balance in plants as reviewed in Jones and Vogt (2001) and Bowles et al. (2005). Furthermore, dimers and polymers, of which *trans*-ε-viniferin is a prominent example seem to be involved in defense mechanisms of plants (Langcake, 1981; Eisenmann et al., 2019). Therefore, enzymes which add sugars, such as glycosyltransferases or enzymes potentially involved in polymerization, such as oxidases and laccases were chosen for further analysis alongside two ABC-transporters. The transporters were chosen in order to include the important mechanic of stilbene transport into the study. This of course does not exclude other types of enzymes and transporters, as well as even transcription factors from the stilbene pathway, but in order to keep the project manageable in time, resources and manpower, this pre-selection was made.

Microarray analysis is often at risk to yield false positive results (Pawitan et al., 2005). To verify the candidate genes identified via microarray experiments, their gene expression was analyzed in VvMYB15 overexpressing grapevine tissue by qPCR Figure 4. While the total values of fold change in the microarrays and relative expression in the qRT-PCR tests differed to some extent, the trends were largely correlating, meaning higher expression in the *VvMYB15* overexpressing tissues relative to the respective control samples. *VvMYB15*, as positive control, can be accepted as successful without much doubt due to the very clear differences between expression and control samples. The stilbene synthase primer tested here (*VvSTS29*), due to the nature of the stilbene synthase enzyme family, is not able to distinguish only the one STS, but rather recognizes *VvSTS25*, *VvSTS27* and *VvSTS29*. It is possible for the three genes to not be part of the incomplete probesets of either of the microarrays. This might explain, why the signal was not present in microarray A, but was detected, as expected, to be upregulated by *VvMYB15* in all tested tissues. The increased gene expression of *VvMYB14* (Figure 4 K) in tissue with elevated *VvMYB15* levels was surprising, since it was shown before that *VvMYB15* repressed the *VvMYB14* promoter (Höll, 2014). Preliminary investigations revealed potential MYB-binding sites in the promoter of *VvMYB15* (Dr. Katja Machemer-Noonan, personal communication) and

previous publications showed some crosstalk between our TFs and other TFs such as *VvMYBA* (Höll, 2014) or WRKY transcription factors (Vannozzi et al., 2018). Further investigations of these interaction an in general of the upstream regulations and players of the stilbene pathway could yield valuable information for a deeper understanding of the synthesis and also roles of stilbenes in grapevine. It was observed that transgenic plants and cell lines with high *VvMYB15* expression levels and thus high stilbene levels were not able to survive (Höll, 2014). Therefore, plants with medium to low *VvMYB15* expression levels were used for the microarray analysis (Höll, 2014). This observation resulted in the hypothesis of a negative feedback loop of stilbenes towards *VvMYB14* and *VvMYB15* (Höll, 2014) and is in line with the results of other researchers, who reported resveratrol to initiate a variety of responses, including cell death (Chang et al., 2011). Furthermore, it was shown that resveratrol had adverse effects in animal cells at higher concentrations (Abbott et al., 2010). Another consequence of this might be the variance between the transgenic plant or hairy root lines transformed with *VvMYB15* or the control construct (Figure 4). Since a pool of cDNA from three lines were used for each, overexpressing and control measurements, it is possible that one line shows a stronger expression than others and thus it was considered a confirmation of elevated candidate gene expression when at least one line showed a clearly increased expression of the candidate gene, which is the case for all candidate genes investigated. Only the two putative stilbene oxidases and both glucanases are not displayed here. Their measurement did not succeed initially and after the following experiments they were deemed secondary candidates and the measurement was not established, respectively repeated due to time constraints.

In order to circumvent the dying of plants that were transformed with the transcription factors, it was attempted to generate plant lines with inducible VvMYB14 and VvMYB15 TFs. Dr. Janine Höll and students started cloning the TFs (and a control construct) via the Greengate cloning system (Lampropoulos et al., 2013), using a dexamethasone inducible expression system. During this thesis project, the transformation, sequencing and selection work was continued. The advantages of this approach are that the plant lines, or also cell cultures or hairy root cultures in the future, could grow without the impairment of high stilbene levels, while still producing the designated TF. Only upon induction before the experiment, the TF would move into the nucleus and the target genes would be activated or repressed (Aoyama and Chua, 1997; Yamaguchi et al., 2015). With plant material modified in this way, it would be possible to utilize optimally expressing plants thus potentially yield further candidate genes, especially with microarrays or RNAseqs that cover more of the *V. vinifera* genome in the future. The transgenic plant lines containing these constructs, are generated from transformed embryogenic grapevine cell lines, produced together with Dr. Günther Buchholz at the AlPlanta (Neustadt an der Weinstraße) according to previous publications (Bouamama et al., 2007; Bouamama et al., 2009). Since the establishment of such plant lines is very time-consuming and needs great

expertise, thorough tests of the constructs are necessary before taking the step into these cell lines. Therefore, the constructs were first used for the transformation of *Nicotiana benthamiana* plants in order to test the correct assembly of the construct parts and the overall localization, both via the mCherry signal. As positive control, the Schumacher group (COS Heidelberg) provided an *Arabidopsis thaliana* VHA-a1-mCherry construct (Lupanga et al., 2020). This control construct showed strong mCherry signals at the expected locations (Figure 5), while the *VvMYB15*-construct showed only minimal, if any, mCherry signal in the cytosol and no change after dexamethasone induction. Either the constructs did not work as expected, which would necessitate a new cloning strategy, probably with a new cloning system, or the expression in tobacco presented some problems itself. It is possible that the protocol simply needs to be optimized for the chosen constructs, or that tobacco as a different plant expression system than the native grapevine, causes the *VvMYB15* expression to work suboptimal.

Nevertheless, the inducible TF approach is very promising and should be continued in the future of the project in order to find more candidate genes.

3.1.2 *In silico* analysis potentially placed *VvLAC* in the defense reaction with stilbenes

The *in silico* analysis of the candidate genes for modification or transport of stilbenes in grapevine was the first step in determining their possible roles after their identification. The genes were analyzed concerning their phylogenetic relations to other enzymes of known function or belonging to similar pathways, as well as concerning their potential intracellular localization via signaling sequences in their amino acid sequence, all with a focus on the most interesting candidates.

In regard to enzymes that are potentially involved in the polymerization of resveratrol, *VvLAC* as member of a gene family shown to be involved in e.g. polymerization of flavonoids (Pourcel et al., 2005), was analyzed in comparison to laccases of known function of several species, from plants, fungi and bacteria. For a meaningful analysis of potential function via phylogenetic relationship, enzymes are needed that are already characterized in similar pathways, as well as from pathways with completely different functions. While for all enzyme categories chosen here, enzymes from the same families of many plants are known, their functions are often not yet characterized, especially in plants that are not model systems. Therefore, this phylogenetic study was conducted with characterized enzymes and predicted ones alike. The *VvLAC* candidate gene is on the one hand grouped closest to a laccase like enzyme that was shown to be involved in oxidation of proanthocyanidins in Arabidopsis thaliana (AtTT10) (Pourcel et al., 2005) and on the other hand distinctly grouped apart from many

laccases that have been associated with either lignin metabolism (*MsLAC1*) (He et al., 2019) directly, or the xylem and potentially indirectly with lignin synthesis there (Figure 6 A). This allows the conclusion that *VvLAC* might be involved in flavonoid or stilbene modification. Furthermore, viniferin is associated with defense responses (Langcake, 1981; Eisenmann et al., 2019), which supports the findings that *VvLAC*, its potential producer, seems to be either localized at the chloroplast or the outside the cell membrane, in the extracellular space (Table 3). Both locations are the site of defense reactions or pathogen sensing. The chloroplast is involved in the synthesis of phytohormones with roles in plant defense, is a producer of reactive oxygen species and important in calcium signaling (Padmanabhan and Dinesh-Kumar, 2010; De Pinto et al., 2012; Lu and Yao, 2018). The plasma membrane as a site for *trans*-ε-viniferin production makes sense when taking into account that the first perceptions, signaling and also like defense reactions occur there as the location of pathogen-plant contact (Nürnberger and Scheel, 2001; Jones and Dangl, 2006; Boller and Felix, 2009). *VvPPO*, *VvStOXY1* and *VvStOXY2* show similar trends in location, but phylogenetic analysis would need to aim at more specific oxidases, which should be done if they are investigated further in the future. Taken together, this information point towards *VvLAC* and *trans*-ε-viniferin being involved in active defense reactions together.

The next group of candidate genes, the glycosyltransferases (*vvGT2*, *VvGT4* and *VvGT5*), were analyzed by phylogenetic- and signal sequence analysis as well, unfortunately with less conclusive results than the laccase analysis (Figure 6 C). The problem here is, that in addition to not many enzymes being thoroughly characterized, glycosyltransferases often have a wider substrate specificity (Jones and Vogt, 2001), potentially making a grouping by functions more challenging than with the laccases previously. One example is the candidate gene *VvGT2*. During *in silico* analysis, it became apparent that the *VvGT2* had already been discovered by other researchers and characterized in at least three publications, although only one was from *Vitis vinifera*. Khater et al. (2012) identified three GTs, the *GT2* in their paper being the *VvGT2* of this thesis, that they characterized as hydroxybenzoate/hydroxycinnamate glucosyltransferases co-expressed with genes related to proanthocyanidin biosynthesis. The material in that study was cloned from *V. vinifera*, cv. 'Macabeu', which is a local variety in northern Spain or southern France. The nucleotide sequence of the gene is completely identical. Hall and De Luca (2007) analyzed a bi-functional *trans*-resveratrol/hydroxycinnamic acid glucosyltransferase of Concord grape (*Vitis labrusca*), which has several single nucleotide polymorphisms (SNPs) compared to the *V. vinifera* gene and Kiselev et al. (2017) investigated a *V. amurensis* version which also showed some SNPs. They associated the gene with stilbene biosynthesis. Especially Hall and De Luca (2007) as well as Khater et al. (2012) showed that the enzyme is not specific to one substrate, but can rather use substrates not only from one pathway, but from related or upstream ones, even depending on the pH of the reaction medium. This,

and the mixed results of localization sequence analysis (Table 3), which might hint to co-localization with specific pathway steps (Jones and Vogt, 2001), led to the conviction that, although the phylogenetic analysis did not yield interpretable clues as to the potential substrates of our glycosyltransferases, they are still worth pursuing further in order to investigate their potential involvement with stilbene glycosylation. The signal sequence analysis of both, VvABC12 and VvABC13, placed them at the plasma membrane (Table 3), which would suggest that they could be involved in stilbene transport into the apoplast, for example to supply the laccases there with resveratrol for dimerization to viniferin within the framework of a defense response. The phylogenetic analysis (Figure 6 C), showed that VvABC13 is closest related to two transporters associated with anthocyanidin transport (Goodman et al., 2004; Francisco et al., 2013), while VvABC12 shows some similarity to transporters predicted to be involved in ginsenoside or salicylic acid transport (Stein et al., 2006; Zhang et al., 2013). While these are good indications, this is not a complete set of V. vinifera transporters, due to the fact that not all proteins are characterized yet.

3.1.3 Promoter induction assays confirmed the candidate genes to be part of the *VvMYB15* regulatory network

The next step after microarray- and in silico analysis was to confirm the activation of the candidate genes promoters by VvMYB15 via dual luciferase promoter induction assay. The transcription factors VvMYB14, VvMYB15, VvMYBA and VvMYBA2 (TFs of the anthocyanidin branch of the flavonoid pathway (Kobayashi et al., 2002; Walker et al., 2007)) as well as a negative control (no construct) were transformed into *V. vinifera* cv. 'Chardonnay' cell culture by particle gun bombardment along with the promoters. Activity was then measured by the luciferase signal of the gene attached to the promoters. The VvSTS29 promoter was used as a positive control (Figure 7 A), since it has been shown to be induced by VvMYB14 and VvMYB15 before (Höll et al., 2013; Höll, 2014). Compared to the values from this previous project, the induction of VvSTS29 by VvMYB15 is in the same fold-range of about 4-fold. As pointed out by Höll et al. (2014), this is a low induction, when compared with other TF/promoter combinations from e.g. different flavonoid pathway branches or upstream genes (Czemmel et al., 2009). This could be attributed to a background activity of *VvSTS29* caused for example by stress, environmental influences such as light or temperature, or phytohormones present in the grapevine cell culture (Lijavetzky et al., 2008; Xu et al., 2012; Höll, 2014; Friedel et al., 2016). All other tested promoters, if induced, showed similar relative induction levels, which could point to the background activity also extending to these promoters, thus originating from the same regulatory mechanics, likely involving the investigated transcription factors. Of the glycosyltransferases, only the *VvGT4* promoter

results are available in this study (Figure 7 B). It is induced by both VvMYB14 and VvMYB15 but not VvMYBA2, thus likely placing it under the influence of the MYB-stilbene regulatory network, just as VvSTS29. The promoters of *VvGT2* and *VvGT5* were unfortunately cloned from an unidentifiable *Vitis* cultivar. The genes and promoters were sequenced and while the genes themselves have almost no differences (2-3 SNPs), the promoters contained more. Still, similar promoter induction experiments were performed on the two promoters, using VvMYB15 as TF and got fold inductions in the same order of magnitude as *VvGT4* and *VvSTS29* (Dr. Katja Machemer-Noonan, personal communication). Of course, this must be repeated in the future, to exclude that any of the changes in the nucleotide sequence leads to different induction or even gene function, since this may well vary between different cultivars. The laccase investigated in this study shows an induction by both TFs (Figure 7 C), although slightly higher by VvMYB15 than VvMYB14. This is a hint towards potential different roles of the two transcription factors, for example one being more active in the defense reactions, while the other one could manage the constitutive gene expression of the stilbene biosynthesis and modification pathway. While interesting, this was not part of this thesis-project, but rather of the investigations of the TFs themselves and their upstream partners and signals, which was begun by Dr. Katja Machemer-Noonan. Of the two ABC-transporters, only *VvABC12* was investigated here, due to initial difficulties in cloning of the *VvABC13* promoter and subsequent time constraints. The *VvABC12* promoter is barely induced by VvMYB15 and VvMYBA2 (1.5-fold, Figure 7 D) and only about 2.5-fold by VvMYB14. This does not necessarily exclude the gene from the list of candidates however, since both ABC-transporters were initially found in *VvMYB15* overexpressing tissues and might still be involved in the stilbene pathway in other cultivars or tissues. Alternatively, they might also be unspecific transporters that are not directly under the control of our two MYB-TFs, but rather act in the wider stilbene-flavonoid-field and be controlled by other related TFs. The last two genes displayed in Figure 7 E + F are the two glucanases (VvGLUC1 and 2). Glucanases have been shown to be induced in *V. vinifera* by *Plasmopara viticola* before (Mestre et al., 2017) and could be for example involved in the release of *trans*-resveratrol from its potential storage form *trans*-piceid by separating the glucose from the *trans*-resveratrol structure. Therefore, it is very promising that they are also induced by VvMYB15 (2-3-fold, respectively) and can be considered candidates for further investigations, although they were not focused on in this study. Furthermore, they are interesting targets of VvMYB15 because glucanases have been shown to act in various ways in defense and stress responses as reviewed in (Balasubramanian et al., 2012) and therefore might have correlating function with stilbenes.

3.2 Gene expression – stilbene level correlations indicated an active defense sole for *VvLAC* and a role in the intracellular stilbene equilibrium for *VvGT2* and *VvGT5*

Following the previous part of this study, where candidate genes were identified, confirmed as regulated by *VvMYB15* (and *VvMYB14*) and first indications on their function were analyzed, this second part aimed to look deeper into the connection of the candidate genes with their respective predicted modified stilbene products. For this approach, the knowledge of differential metabolite accumulation in grape berry development (Figure 2) as well as during *Plasmopara viticola* infection (Langcake, 1981; Eisenmann et al., 2019) was utilized for an attempt to connect candidate gene expression with metabolite content in separate correlation studies. Furthermore, in regard to the studies by Dr. Birgit Eisenmann, it was attempted to investigate the connection between the role of stilbenes in resistance and the resistance, respectively the resistance mediating loci (Rpv, resistance to *Plasmopara viticola*) by some *Vitis* cultivars.

3.2.1 *VvGT2*, *VvGT5* and *trans*-piceid are likely involved in the putative phytoanticipin function of stilbenes via storage or detoxification role

The contents of a grape berry are of vital importance to the quality of the wine produced from it and thus, much effort has gone into analyzing the amounts of different metabolites from berry onset to the ripe berry (Conde et al., 2007). Naturally, these changes in metabolite levels must be accompanied by changes in gene expression of the enzymes needed to produce the metabolites and therefore need to be affected by the regulatory network controlling them. As other studies in the field of grapevine metabolism have shown, it is possible to gain insights into the relation of candidate genes with metabolite levels and from there also conclude many other information (Hall and De Luca, 2007; Sweetman et al., 2009; Höll et al., 2013). In this study, *V. vinifera* cv. 'Pinot Noir' berries were sampled over the course of one growing season (19 weeks) and analyzed for the gene expression of our candidate genes on the one hand and for the content of selected stilbenes on the other hand. The expression of *VvMYB14*, *VvMYB15* and *VvSTS29*, as well as the *trans*-piceid content of a comparable developmental series was measured in the previous project (Höll et al., 2013; Höll, 2014). Then and now in this study, *VvMYB15* and *VvSTS29* showed a strong increase in relative expression levels after vérasion in week 11, as well as little higher expression in week one as compared to the following weeks until vérasion (Figure 8 A + B). The corresponding stilbene in this case is *trans*-resveratrol, which is the

first stilbene in the biosynthetic pathway and the product of the stilbene synthase (*STS*) gene family (Figure 9 A). *Trans*-resveratrol corresponded well to the two genes, with an initial peak at week one and increased amounts after week 11. The initial peak was relatively large when compared to the later levels and can possibly be explained by the dilution effect, since the volume of the grape at this stage is much smaller than in the late ripening stages. Nevertheless, an initial protective effect for the young, vulnerable berries against e.g. *Plasmopara viticola* infection, which can occur in the vineyard (Fröbel and Zyprian, 2019) would certainly increase the survivability.

Trans-piceid, the glycosylated form of *trans*-resveratrol, which is very prominent in grapevine, showed a similar accumulation pattern over the course of a season as was already determined earlier (Höll et al., 2013) (Figure 9 B). Compared to *trans*-resveratrol, the ng/gram freshweight level was 10 times higher, while following the same pattern of high accumulation in week one and after véraison. Of the candidate genes suspected to be responsible for the modification of *trans*-resveratrol to *trans*-piceid, the three glycosyltransferases, only *VvGT2* and *VvGT5* showed an expression in qRT-PCR analysis that resembled the expression patterns of *VvMYB15* and *VvSTS29* (Figure 8 C + E) and piceid (Figure 9 B), indicating a correlation between candidate gene and stilbene product. These constitutively higher *trans*-piceid than *trans*-resveratrol levels, which might be caused by the increased expression of the two GTs, point towards a phytoanticipin function (VanEtten et al., 1994), which involves *trans*-piceid either as storage reservoir or as detoxified compound. This *trans*-piceid background level has already been shown in other studies (Jeandet et al., 1992; Versari et al., 2001; Gatto et al., 2008; Eisenmann et al., 2019). Glycosylation reactions can have a wide range of functions, but broadly can be attributed with stabilization, detoxification and solubilization, leading to transport for example into the vacuole (Jones and Vogt, 2001; Dima et al., 2015; Le Roy et al., 2016). Either *trans*-piceid is stored there in order to keep a *trans*-resveratrol homeostasis in the cell, enabling a quick response upon damage or infection, or the *trans*-piceid might even serve as reservoir and be reactivated upon such situations by cleaving of the glucose and transport towards the defense location. This would also explain the similar expression patterns of *VvABC12* and *VvABC13* (Figure 8 G + H), which could be involved in the transport of the glycosylated compounds, although their initial localization sequence prediction places them at the plasma membrane (Table 3). This should be among the first things investigated in vivo when focusing on the transporters in the future, since there is evidence for involvement of ABC-transporters, possibly with involvement of glutathione conjugates, in the vacuolar transport of e.g. glycosylated phenylpropanoids (Marrs et al., 1995; Larsen et al., 2003; Kitamura et al., 2004; Francisco et al., 2013) and even differential transport across vacuolar and plasma membranes depending on the glycosylation state (Miao and Liu, 2010). *VvGT4* on the other hand showed no such trend, but rather that its maximal expression in week two and fell from there on (Figure 8 D). This does not exclude the candidate from

consideration as a possible stilbene-GT, but might indicate that it acts in other tissues or functions yet to be discovered.

Trans-ε-viniferin shows a similar pattern of accumulation as both previously investigated stilbenes, but in much lower amounts (up to 300-400 ng/g freshweight, Figure 9 C), while *trans*-pterostilbene was not detected in the samples at all. Both stilbenes were associated with an active defense response (Langcake, 1981; Schmidlin et al., 2008; Eisenmann et al., 2019) rather than a constitutive accumulation in the cells. This was also the indicated by the expression levels (Figure 8 F + I) which do not correlate with either *VvMYB15* or *VvSTS29* during development and the stilbene levels of their respective (predicted) products, *trans*-ε-viniferin and *trans*-pterostilbene.

Concerning the stilbene analysis via HPLC, the compounds were chosen firstly according to their importance in grapevine as previously published (Langcake, 1981; Jeandet et al., 1992; Gatto et al., 2008; Schmidlin et al., 2008; Höll, 2014; Eisenmann et al., 2019) and only secondly due to the availability of standards for identification and quantification. Only one analysis with technical replicates is depicted in this study, since another measurement was performed on a different HPLC device, which showed similar trends, but due to other specifications and sensitivity, the results were not calculated together (data not shown).

3.2.2 Correlation between *VvLAC* expression and *trans*-ε-viniferin accumulation indicated a role for both in active defense

While the developmental series analyzed before covered 19 weeks of growth and development, the investigation there did not focus on special situations, which can strongly influence the stilbene levels. This can present a problem especially for the more active or toxic compounds, since they are likely not constitutively present in high or even detectable levels (Chang et al., 2011). Therefore, this next experimental series was designed to investigate the candidate gene expression and corresponding stilbene levels during a response to infection. The experiment was conducted in cooperation with Chantal Wingerter. The plant material chosen was *V. vinifera* cv. 'Regent' which has an Rpv3 locus, giving it some resistance to *P. viticola,* and *V. vinifera* cv. 'Pinot Noir' without known resistances to *P. viticola*. The infection experiment was performed on both cultivars with *P. viticola* spores (infected group) and water (mock group) and samples were taken after 0 h, 6 h, 12 h, 24 h and 48 h. The measured expression of *VvMYB15* and *VvSTS29*, as two essential genes and thus positive controls, indicated the highest expression levels in the 'Regent' infected samples, with a peak in relative expression at 6 hpi and 24 hpi (Figure 10 A + B). The levels of *trans*-resveratrol showed a corresponding trend, with the maximal amount present at 48 hpi (Figure 11 A), which was observed in a previous

study as well (Eisenmann et al., 2019). Also showing similar trends as in this previous study were the two stilbenes *trans*-ε-viniferin and *trans*-pterostilbene (Figure 11 C + D). Trans-pterostilbene was only present in the 48 hpi 'Regent' infected sample, which correlated well with the gene expression of *VvROMT*, confirming its role as *trans*-pterostilbene producer (Schmidlin et al., 2008) as well as active defense compound (Langcake, 1981; Pezet et al., 2004a). A similar trend was visible in the correlation of *VvLAC* expression and *trans*-ε-viniferin accumulation, which both showed some background in mock and 'Pinot Noir' samples, but had their peaks in the infected 'Regent' samples at 48 hpi. The backgrounds could be the effects of other stress factors, such as the mechanical wounding or prior infections during growth in the greenhouse. Nevertheless, this is a strong indication that *VvLAC* might be involved in *trans*-ε-viniferin production within the framework of a defense reaction against *P. viticola*. The last stilbene that was investigated in this study, *trans*-piceid, did not show any clear trend towards an increase during infection or a higher amount accumulating in 'Regent' than 'Pinot Noir' (Figure 11 B). Only the 48 hpi 'Regent' infected sample was slightly higher than the other samples, but this might well be the elevated *trans*-resveratrol levels that are metabolized. The three glycosyltransferases showed no higher expression during infection or in 'Regent' and no clear pattern is recognizable between them (Figure 10 C - E). The same holds true for the two ABC-transporters (Figure 10 E+F). This indicates that, as discussed before in context of the developmental series, *trans*-piceid and its associated enzymes, do not play an active role in defense, but are rather there for constant storage or detoxification. The levels of *trans*-resveratrol in the study of Eisenmann et al. (2019) were comparable to the ones in this project, as were the levels of *trans*-pterostilbene. This was not the case with the other investigated stilbenes, *trans*-piceid and *trans*-ε-viniferin. For these two compounds, the levels were three times higher (*trans*-piceid) and over ten times higher (*trans-ε*-viniferin) on a ng/g freshweight level in the previous study. The 3-fold increase in the *trans*-piceid levels could be explained by differences in handling and measurement, but the 10-fold increase in *trans*-ε-viniferin demands another explanation. This could be found in each year's newly harvested strain of *P. viticola*, since this was observed to have an effect on the infection and stilbene levels, even though it as always taken care to collect strains that were not previously exposed to resistant plants (personal communication B. Eisenmann, C. Wingerter). Nevertheless, the experiment showed a promising correlation between laccase expression and *trans*-ε-viniferin accumulation in the context of an active defense response, which led us to include the candidate gene in further experiments, along with the glycosyltransferases for their more "passive" function.

3.2.3 Initial investigations on stilbenes during the development of different Rpv-containing cultivars indicated more potential in infection-series experiments on Rpv loci

In light of the promising results of the infection series in two different cultivars with and without a known resistance to *P. viticola*, the question arose whether this correlation between candidate gene and potential stilbene product would also be observed in other *V. vinifera* cultivars with different resistance mediating Rpv-loci during grapevine development. In addition to that, it was also investigated, whether such differences between the cultivars were also observable during development of the respective grape berries. Therefore, in spring, the inflorescences of 'Muscaris' (Rpv10), 'Calardis Blanc' (Rpv3.1 + 3.2), 'Cabernet Blanc' (Rpv 3.1), 'Cal6-04' (Rpv 3.1 + 12) and 'Riesling' (no Rpvs) were collected, as well as their ripe berries in fall. These materials were chosen to get a first impression of the candidate gene – stilbene correlation in the two extreme states of development, and potentially base further, more extensive studies on the results. An extensive infection series with multiple cultivars was deemed to be not the main focus of this study and thus too time and resource consuming. As discussed before, Eisenmann et al. (2019) focused their work on Rpv3, where they also did extensive analysis, including RNAseq, in order to elucidate the role of stilbenes in Rpv3. While they showed a very strong correlation between stilbenes, Rpv3 and plant resistance, the connection of the genes in the loci (Foria et al., 2020) to the stilbene pathway remains yet to be identified.

Unfortunately, RNA extraction for the grape berry samples was not successful, neither via several commercially available kits, nor by more complex, manual methods (Hot-borate extraction). This can be most likely be attributed to the extremely late sampling timepoint (days before commercial harvesting), since the metabolite content, especially polyphenols are highly enriched by that point, which complicates RNA extraction (Birtić and Kranner, 2006; Vasanthaiah et al., 2008).

The analysis of the transcript levels of the candidate genes in the inflorescence material Figure 12 and stilbenes levels in both Figure 13, inflorescences and berries yielded no readily interpretable tendencies, neither concerning the correlation between gene expression and product, nor between the cultivars. While this was only a first glance and can be repeated in follow up studies, possibly also with a timepoint at véraison as well as a more solid RNA extraction method, the approach of a more extensive infection series should be more promising, since after all, the Rpv loci genes are associated with functions in grapevine defense by definition. Information on either stilbene-related genes in grapevine, or the exact composition of Rpv loci, maybe even stilbene-related genes in the Rpv loci, would be valuable information for future breeding attempts, since genetically modified crop plants are not currently well-received or even allowed in the European Union and Germany.

3.3 Yeast and bacterial expression systems showed *trans*-resveratrol to be a substrate for VvGT2

In the two previous parts of the study, the candidate genes were filtered by investigations on their interaction with VvMYB15, as well as database information in part one and by correlation studies in developing and infected *V. vinifera* plant material in part two. In this third part of the thesis, four of the most promising candidate genes were to be further characterized biochemically and it was attempted to prove that they directly produce certain stilbene products.

In order to achieve this goal, *VvGT2*, *VvGT4*, *VvGT5* and *VvLAC* were first expressed in a yeast strain that is capable of producing grapevine resveratrol, then expressed in a bacterial system for protein extraction and enzymatic assay and finally, *VvLAC* was expressed in a tobacco system with the same purpose, but with the aim of improved expression in a eucaryotic, respectively plant system.

3.3.1 VvGT2 is able to use trans-resveratrol as substrate for trans piceid production

Saccharomyces cerevisiae cultures, among others, have been used in several studies on the biotechnological production of stilbenes and related molecules in the past years (Becker et al., 2003; Beekwilder et al., 2006; Zhang et al., 2006; Vannelli et al., 2007; Xue et al., 2007; Katz et al., 2013). Two *S. cerevisiae* strains, equipped with the molecular tools to produce resveratrol, taken from *V. vinifera*, were provided by Evolva Holding SA. for research purposes (Katz et al., 2013).

This was used to potentially reach two goals. First, an expression system such as yeast, which in a setup with the Gateway cloning technology is transformable very quickly, would be an invaluable quick check system for potential candidate genes, without the need for protein extraction and in vitro assays. Second, in case of successfully characterizing a candidate gene as stilbene-modifying, with this and other methods, there would also a first production tool implemented, which could then be refined, making it also interesting for the company.

The yeast culture itself provides two measurable benchmarks for a functioning system. The density of the culture was measured as OD_{600}, which showed some differences in culture growth, but overall, the cells were able to grow as expected. In the initial trials, this was not the case, since it seemed that galactose, which was needed for induction, as sole carbon source was not sufficient for reliable growth in liquid culture. After addition of raffinose to the media, the growth was reliable and reproducible, within the fluctuations as depicted in Supplemental Figure 2. With some more work on fine-tuning the

culture media and growth conditions, this indicated a reliable system. The second benchmark is the *trans*-resveratrol content, which is produced in all cultures, regardless of induction of the additional plasmids. *Trans*-resveratrol was measured in six of the eight cultures depicted in Figure 14 in quantities between 1,000 and 3,000 ng/ml culture. These fluctuations as well as the two either non-producing or non-measurable cultures indicate that some improvement work has to be put into the project in the future, but overall, it is a good system for the first quick-checks undertaken here.

Of the candidate genes investigated with the yeast-expression-system, a modified stilbene was detected only in the *VvGT2* containing, induced culture. There, *trans*-piceid was measured with 46 ng/ml culture (Figure 14). This is in line with the results from the enzymatic assay (see next chapter, 3.3.2), where VvGT2 also was the only producer of the glycosylated *trans*-resveratrol from the available candidate genes. It remains to be seen, whether higher levels of *trans*-piceid production would be possible when time is put into the yeast-expression-system optimization, or whether even the other glycosyltransferases are able to produce *trans*-piceid, respectively the laccase to produce *trans*-ε-viniferin, under optimized conditions.

3.3.2 VvGT2 and VvGT5 likely do not use *trans*-resveratrol as main substrate but could still fit into the stilbene modification pathway as typically unspecific glycosyltransferases

The expression in the yeast system provided first insights into a possible production of modified stilbenes by the candidate genes as well as a quick check method for future candidate genes. A more thorough, but also more complex and time-consuming approach is the extraction and possible purification of the enzymes from an expression system with a subsequent *in vitro* enzymatic assay with specific substrate combinations. This enables the calculation of kinetic data for separate enzymes and a broad scan of possible substrates. Researchers have used this approach for example in the discovery of the, so far, only known stilbene modification enzyme, the resveratrol-o-methyl transferase (Schmidlin et al., 2008) as well as in the analysis of grapevine glycosyltransferases, including the VvGT2 of this study, as described earlier (Hall and De Luca, 2007; Khater et al., 2012). While the advantage of this method over the quick yeast-expression system is more detailed information on the enzymatic reaction and more controllable reaction conditions, a clear drawback is the inherent difficulty of enzyme expression and extraction or purification under non-denaturing conditions. In this experiment, only *VvGT2* and *VvGT5* were investigated. VvGT4 proved impossible to clone into the necessary vector within the timeframe of the experiment, due to a fixed date for travel to Dr. Hugueneys laboratory where the experiment was performed and later restrictions due to the pandemic in the year 2020.

VvLAC was successfully cloned into the appropriate vectors, but expression, even in various bacterial strains and under different conditions was not successful. Therefore, this enzyme was later investigated in *N. benthamiana* (chapter 3.3.3 and Supplemental Figure 6) in order to reap the benefits of an eucaryotic expression system. For this experiment, VvGT2, VvGT5 as well as At73B5, an *Arabidopsis* glycosyltransferase with functions in resistance reactions (Lin et al., 1999; Langlois-Meurinne et al., 2005) that is used as a positive control due to its acceptance of a wide variety of substrates, were expressed in BL21 (DE3) *E. coli* cells. The crude extract of cells with one of the genes, induced or uninduced or from empty control cells was then used for an enzymatic assay with 28 different substrates as potential targets for glycosylation by the enzymes. The focus here was on *trans*-resveratrol, kaempferol (flavonoid) and esculetin, which is a coumarin derivative, also found in grapevine (Goufo et al., 2020) and a widely glucose acceptor in enzymatic assays of this kind (Dr. Hugueney, personal communication).

With no tags, or only a his-tag for later purification resulted in near 100 % precipitation (data not shown), which was circumvented by expression with a NusA tag for better solubility (De Marco et al., 2004). With these constructs, the solubility was increased enough to have sufficient protein for the assay in the soluble fraction of the crude extract (Figure 15).

The original plan for this project part was to get a first idea about the enzyme activities from the crude extract and a broad mix of substrates and then, in a second step, refine the method, establish a reliable purification protocol, e.g. via the included his-tags, as well as quantification of the substrate and products and lastly, calculate the enzyme kinetic parameters from the measurements. As mentioned above, travel and work restrictions prevented the full execution of the project, but nevertheless, the measurements made with the crude extracts allowed good insight into the activity of the two glycosyltransferases.

Due to the mentioned missing time for quantification, the results in Figure 16 are displayed as peak areas of the substrate relative to the control sample. Furthermore, the peaks of the two different masses cannot directly be compare due to the different “response factor” of the molecules in the mass detector. Therefore, the ability of an enzyme to use a substrate for glycosylation was analyzed by checking whether or not the product was found in the sample and if so, checking by how much the original substrate peak was reduced compared to the non-enzyme control, respectively the other peaks, as is depicted exemplary in Supplemental Figure 4and in an overview in Figure 16.

First, it was evident that *At73B5* seemed to be able to convert all of the three displayed substrates into their respective glycosylated forms, with kaempferol being almost completely metabolized, esculetin to about 50 % and even *trans*-resveratrol was used well as glucose acceptor. The *trans*-resveratrol control cell line showed a comparatively low amount of substrate and most likely needs to be re-measured. In the *VvGT2* samples, substances with the exact masses of the glycosylated products were

found in all three cases, although to a much lower extend. Here, also kaempferol was metabolized with the highest rate, followed by esculetin and *trans*-resveratrol with much lower rates. Similarly, in the publication of Hall and De Luca (2007), kaempferol was metabolized faster than esculetin by the *V. labrusca* GT2, although *trans*-resveratrol showed a much lower rate in our experiment. As Hall and De Luca (2007) and Khater et al. (2012) found out in their studies, and preliminary experiments indicated also in this study (results not shown), the reactions of the enzymes are strongly pH-dependent. The best results for *trans*-resveratrol and flavonoids were observed at pH 9, while our experiments were conducted at pH 7.5. Therefore, it can be suspected that more extensive trials, as initially planned, would give a better picture of the abilities of VvGT2 and the other enzymes. VvGT5 was the candidate with the lowest rates of metabolization. It did not accept *trans*-resveratrol as a substrate in detectable amounts, barely used esculetin and metabolized Kaempferol very minimally.

The fact that *VvGT2* and to a lower extend also *VvGT5* used kaempferol and esculetin more efficiently than *trans*-resveratrol, or in the case of *VvGT5* seem not to use *trans*-resveratrol at all, put into question of their role as stilbene modifying enzymes. This could simply be a result of sub-optimal conditions during protein extraction or within the assay, especially since Hall and De Luca (2007) found a much higher efficiency for *trans*-resveratrol metabolization, depending on pH. Thus, optimization in the experimental procedure is still needed. On the other hand, many GTs are not very substrate specific, but rather glycosylate a wide variety of substrates either in general (Leah et al., 1992; Pflugmacher and Sandermann Jr, 1998; Kramer et al., 2003; Modolo et al., 2007), localization-specific (Vogt et al., 1997; Kramer et al., 2003; Yonekura-Sakakibara et al., 2007) or after recognition of some common structures (Marcinek et al., 2000). This non-specificity has also been observed in the expression experiments with tobacco (results not shown), where any added *trans*-resveratrol was quickly and efficiently metabolized to *trans*-piceid as long as UDP-glucose was available. Since a regulation by *VvMYB15* was confirmed in chapter 1 and very good correlations between candidate gene expression and *trans*-piceid content were found in chapter 2, this result throws off this line of results to a certain extend. One explanation might be found in the evolutionary history of the stilbene pathway itself. Since the stilbene synthases evolved from chalcone synthases (Schroder et al., 1988b; Tropf et al., 1994), which are responsible for the flavonoid pathway, it might well be that also other genes downstream of VvMYB15 are adapted or lend from other pathways. In this case it would of course be of great interest to find said pathways and original substrates.

To this end, a mix of 17 polyphenols and 11 further common substrates was used for the same enzymatic assay experiments with the three enzymes and analyzed with the ToxID software (Thermo Fisher), which enables a simultaneous search for multiple substrate and product masses (Supplemental Figure 5). Table 4 displays four substrates that were found to be glycosylated in the assay by at least one or both candidate genes. Caffeic acid is involved in the phenylpropanoid pathway, myricetin and

naringenin are flavonoids and thus from a closely related pathway and ethyl gallate is the ethyl ester of gallic acid. Further studies with these substrates and also the search of more potential targets of the candidate genes in this way are essential for completing the picture of stilbene modification pathways in the near future.

3.3.3 Simultaneous *VvSTS29* and *VvLAC* expression in *N. benthamiana* yields a slight increase in a yet unidentified substance with a very close retention time similarity to *trans*-ε-viniferin

Following the expression of candidate genes and analysis of their products in yeast and bacteria, the last step was to transfer this concept into plants, where there is a more natural environment for expression and reactions on the one side, but also in general possible improved expression and folding conditions due to the eucaryotic background of the expression system (Netzer and Hartl, 1997). Concerning the expression and analysis of the glycosyltransferases, there are limitations however. Regardless whether the experiment was conducted in a system where *trans*-resveratrol was produced in planta by a *V. vinifera* STS or in an extract with added *trans*-resveratrol, enzymes from tobacco were able to metabolize *trans*-resveratrol to *trans*-piceid efficiently. The same effect was observed in Arabidopsis during preliminary experiments (data not shown) as well as by other researchers, who managed to show glycosylation of substances usually not occurring in a certain plant system (Li et al., 1997; Bak et al., 1999). Therefore, only *VvLAC* was analyzed in this expression system, in the hopes that the laccase would be able to metabolize a detectable amount of *trans*-resveratrol into *trans*-ε-viniferin before unspecific enzymes deplete the resveratrol pool.

In the analysis of the leaf extracts, which were prepared after transformation with either STS, LAC, both or neither, and an incubation time at greenhouse conditions for two to four days, it was apparent that no *trans*-ε-viniferin was produced, although a substance with very similar retention time to *trans*-ε-viniferin was noticed. With an elution time only differing only 3-4 seconds from *trans*-ε-viniferin and an increase of peak area not observed in the samples which did not contain both VvSTS29 and VvLAC, the substance might be closely related to *trans*-ε-viniferin (Supplemental Figure 6). An explanation could be as simple as a slightly differing modification in *N. benthamiana* as compared to the same reaction in *V. vinifera*. Of course, this can also be coincidence, but finding out the nature of this compound, e.g. by UHPL/MS, therefore would be helpful in either gaining a promising line of further research or excluding a false one for future research. As with the expression systems before, more work needs to be done towards reliably establishing the method for future candidate genes.

3.4 Results summary and conclusion

This thesis comprised of the ambitious plan to accomplish the complete way from identification to characterization of not only one but several genes that are potentially involved in the biosynthesis of modified stilbenes in *V. vinifera*. While there remains much work to be done with each gene, there have been results pointing towards very promising candidate genes and experimental procedures as well as strategies of filtering the sheer mass of potential candidates have been established.

The microarray of *VvMYB15* overexpressing tissues by Dr. Janine Höll was the foundation of the project and also still holds a great number of potentially interesting genes for future research. With glycosyltransferases, the laccase and other oxidases as well as the two ABC-transporters, a group of genes was chosen representing active and passive factettes of the stilbene biosynthesis pathway and even transport mechanisms. The chosen candidate genes were investigated *in silico* and their phylogenetic relations as well as predicted localizations were as valuable as the confirmation of the VvMYB15-promoter inductions for choosing to involve them in the second and third parts of the thesis project.

The investigations into the correlation between candidate gene expression and present amount of the expected modified stilbene provided valuable additional insights into the potential function of these products. The glycosyltransferases *VvGT2* and *VvGT5* as well as the two ABC-transporters *VvABC12* and *VvABC13* correlated well with the general *VvMYB15* and *VvSTS29* expression levels and *trans*-resveratrol and *trans*-piceid content during grape berry development. This added to the hypothesis of them being responsible for *trans*-piceid production as well as indicating a function in stilbene storage or detoxification in the framework of a potential phytoanticipin role. *VvLAC* and *trans*-ε-viniferin correlated well during infection with *Plasmopara viticola*, suggesting a potential involvement of the candidate gene in *trans*-ε-viniferin production and an involvement of the substance in plant defense reactions, similar to the active defense compound *trans*-pterostilbene with VvROMT as its producer. Furthermore, the potential involvement of stilbenes in the Rpv-loci related defense as investigated by Dr. Birgit Eisenmann was reenforced.

The third part of this thesis, the most important direct characterization of the filtered candidate gene enzymes, although unfortunately cut short by time and pandemic-travel restrictions, yielded valuable first insights into the enzymes abilities of stilbene modification and laid the groundwork for further characterization experiments in the near future. More effort needs to be put into the cloning of *VvGT4*, and the expression and analysis of *VvLAC* in a plant-based expression system. VvGT2 and VvGT5 were analyzed in an enzymatic assay and while VvGT2 was able to metabolize *trans*-resveratrol to a low extend, VvGT5 was not. This might be due to more needed optimization work in the assay or analysis

or due to the fact that *trans*-resveratrol could be an unintended substrate in a pathway only lending the enzyme and the main substrate for the enzymes is yet to be found.

3.5 Outlook

From the initial identification to the first characterization experiments of selected candidate genes much has been accomplished in this project, especially in the establishment of strategy, workflow, techniques and in the analysis of a first, small group of candidate genes. In a project as extensive as finding an almost completely unknown pathway of modification and transport genes, of course there remains much work to be done.

Firstly, even outside of the scope of this project, there remains the question regarding additional players in the transcription factor regulatory network of stilbenes, meaning the search for TFs upstream of VvMYB14 and VvMYB15, as well as other TFs or signal pathways regulating the resource flow towards or from the stilbene pathway in different developmental stages or infection scenarios. This was begun by Dr. Katja Machemer-Noonan and makes for an interesting future project.

The microarrays themselves yet contain a plethora of interesting candidate genes, from enzyme-groups that were upregulated but not chosen for the types of reactions to the so far completely unanalyzed downregulated genes, which might give invaluable hint also towards the transcription factor network by showing which other pathways are downregulated in order to direct the flow of resources towards stilbene production.

As mentioned at several points during this thesis, there of course remains work to be done at every stage of the three parts reported on here. For part one, more genes of promising enzyme, transport or transcription factor groups should be selected for analysis and all genes can benefit from more detailed database analysis and the findings of other researchers.

In part two, the correlation studies can be completed for some genes that were not yet included in the analysis due to time or resource constraints and new candidates can undergo selection here. Also, the connection of stilbenes with the Rpv loci for resistance against *P. viticola* already investigated by Dr. Birgit Eisenmann promise to yield valuable information for potential targeted breeding in the near future.

Part three, biochemical characterization, needs more establishing work in the future and hopefully, the cooperation with Dr. Hugueneys laboratory in Colmar can be re-ignited after the pandemic shutdown and then increased towards the goal of characterizing candidate genes in enzymatic assays and make use of the vast metabolite library for substrate search.

Finally, the setup of a stilbene production system for potential industrial scale production, e.g. in cooperation with Evolva, should not be lost from sight. While the yeast expression system in this study was primarily used as a quick-check-system for candidate genes, a successful characterization of one or the other gene as stilbene modifying suggests a potential production and therefore upscaling of this expression system.

4 Material and Methods

4.1 List of Vit-numbers for identification of the mentioned *V. vinifera* genes

Table 5: List of genes and corresponding Vit-numbers.

Candidate Gene	Vit-No.
VvGT2	VIT_03s0180g00200
VvGT4	VIT_02s0025g01240
VvGT5	VIT_03s0017g02110
VvLAC	VIT_18s0001g00680
VvSTS29	VIT_16s0100g00990
VvMYB13	VIT_05s0049g01010
VvMYB14	VIT_07s0005g03340
VvMYB15	VIT_05s0049g01020
VvABC12	VIT_09s0002g05570
VvABC13	VIT_07s0005g02660
VvStOXY1	VIT_18s0001g11470
VvStOXY2	VIT_18s0001g11430
VvGLUC1	VIT_06s0061g00120
VvGLUC2	VIT_05s0077g01150
VvWRKY03	VIT_01s0010g03930
VvWRKY52	VIT_17s0000g01280
VvJAZ8	VIT_10s0003g03790
VvPAL1	VIT_16s039g01100
VvPPO	VIT_10s0116g00560
ROMT	VIT_12s0028g01880

4.2 Plant material:

VvMYB15 overexpressing lines

The plant material for the microarray analysis and subsequent validation via qRT-PCR was generated by Dr. Janine Höll prior to this project and is described in her thesis (Höll, 2014)

V. vinifera cv. 'Pinot Noir' developmental series

Grapevine berries (*V. vinifera* cv. 'Pinot Noir') for RNA extraction and stilbene level measurement were collected in a commercial vineyard in Schriesheim (Germany, 49° 47' 22" N 8° 67' 09" E) in the year 2011. The climate was moderate with a mean daily temperature of 22 °C at the start of sampling. Samples were taken every one to two weeks, covering the different developmental stages. 100 berries from different plants were collected, mixed, homogenized and directly frozen in liquid nitrogen as described before (Downey et al., 2003; Höll et al., 2013).

V. vinifera infection series

For the infection of leaf discs, *Vitis vinifera* plants were grown under greenhouse conditions. The cultivars 'Regent' and 'Pinot Noir' were chosen for the experiments. 'Regent' is a cultivar resistant to *Plasmopara viticola* (Downy mildew) and *Erysiphe necator* (Powdery mildew) since it contains the resistance loci Ren3, Ren9 and Rpv3.1. It was crossed by G. Alleweldt 1967 at Geilweilerhof, Institute for grape breeding from 'Diana' and 'Chambourcin' and is a red grapevine cultivar (Eibach and Töpher, 2002). 'Pinot Noir' is a traditional red grapevine cultivar that possesses no specific resistances to the mentioned (or other) pathogens and is included as a susceptible control.

The plant material was collected in the vineyards of the Dienstleistungszentrum Ländlicher Raum Rheinpfalz (Neustadt an der Weinstraße, Germany) as cane cuttings. They were soaked in water for one night, then 8 h in 0.5 % Chinoplant® (FMC Cheminova, Germany) for disinfection. The cuttings were stored at 4 °C and 95 % relative humidity during the winter time and used the next spring and summer for cultivation of new plants. The cuttings were chipped again at the basal end, capped in warm wax at the apical end and placed in Perligran® Extra (Knauf AQUAPANEL, Germany) instead of soil for 7-8 weeks at 24 °C in the greenhouse. Subsequently, the cuttings with developed leaves and roots were potted in a grapevine soil mixture containing 90.1 % Floradur® multiplication substrate (Floragard, Germany), 9.0 % Perligran® Extra, 0.5 % carbonic magnesium-lime (Hufgard, Germany) and 0.4 % MANNA® COTE 6 M (Hauert MANNA, Switzerland). The plants were grown in the greenhouse at

22 °C during the day and 18 °C at night with always 50 % humidity. The plants were ready for experiments after 2-3 month (six to eight leaves stadium).

Flower and berry material of different cultivars

Vitis vinifera early flower clusters were collected in spring and ripe berries in fall of the year 2018. The cultivars collected were grown in a trial field near Neustadt an der Weinstraße (Germany, 49° 37’ 29” N 8° 18’ 24” E). Samples were taken from the cultivars ‘Cal6-04’ (‘Sauvignac’), ‘Cabernet Blanc’, ‘Calardis Blanc’, ‘Muscaris’ and ‘Riesling’. One flower cluster was collected from five plants, while berries were collected from multiple clusters of five plants. Both were immediately frozen and later the flowers samples and berries were pooled among themselves and ground to a fine powder.

V. vinifera cv. ‘Chardonnay’ cell culture

The grapevine cell suspension cultures used for the promoter induction assays (dual luciferase assay) were set up from petiole callus culture of the cultivar ‘Chardonnay’, grown to log-phase, filtered and then inoculated at a cell density of 10 % (v/v) in liquid Grape Cormier (GC) medium (Bao Do and Cormier, 1991) as described before (Czemmel et al., 2009).
The embryogenic cell cultures for production of stable transformed wine plants (done by Dr. Janine Höll before this project and here only in planning but not performed) were produced as described before (Bouamama et al., 2007) from anthers of ‘Selektion Oppenheim’ (SO4) cultivar.

Nicotiana benthamiana

Tobacco (*Nicotiana benthamiana*) for leaf infiltration with *Agrobacterium tumefaciens* was grown in standard greenhouse conditions for four to five weeks.

4.3 Bacteria and Yeast strains methods

Bacterial strains

For cloning, selection and plasmid propagation *Escherichia coli* bacteria of the TOP10 strain by Thermo Fisher were used:

F- *mcrA* Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ *lac*X74 *rec*A1 *ara*D139 Δ(*araleu*)7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G

For preparation of plasmids containing the ccDB gene, needed in the Gateway ® Cloning system *E.coli* bacteria of the DB3.1 strain were used:
F- gyrA462 endA1 glnV44 Δ(sr1-recA) mcrB mrr hsdS20(r_B^-, m_B^-) ara14 galK2 lacY1 proA2 rpsL20(Sm^r) xyl5 Δleu mtl1

For protein expression of cloned genes *E.coli* bacteria of the BL21(DE3) strain by New England Biolabs were used:
F^- *ompT gal dcm lon hsdS*$_B$($r_B^-m_B^-$) λ(DE3 [*lacI lacUV5-T7p07 ind1 sam7 nin5*]) [*malB*$^+$]$_{K\text{-}12}$(λ^S)

Agrobacteria

For transformation of *Nicotiana benthamiana* plants, *Agrobacterium tumefaciens* strains C58C1 or EHA105 were used, respectively.

Yeast strains and patent

For expression of candidate genes in resveratrol producing *Saccharomyces cerevisiae* strains, the strains EFSC3497 (parental strain of FS09322 as described in US 2013/0209613 (Katz et al., 2013)) and EFSC3672 (containing similar resveratrol producing genes as FS09322 as described in US 2013/0209613) were used:
EFSC3497: Matalpha ura3-52 his3 Leu2 pTPI-Acc1, DARo10, deltaTRP1, p204 (pTEF-C4H::CYBg::ATR2 pCUP1-PAL2, TY element, URA3), p180 (pTDH3-4CL2 pTEF1-VST1, TY element, S. plombe HIS5-Tag2), Rho51 (TEF1-Snq2, trp1)
EFSC3672: Mat alpha, ho-, KAN, ura3delta, aro10::(AtPAL2<-pTDH3-pTEF2->C4H-Cyb5-ATR2,pTEF1->ACC1**,VvVST1<pTPI1-pDC1->At4CL2,-DR), XII-*1::(pTEF1-ACC**, C4H:b5:ATR2<-pTEF2-pTDH3->AtPAL2, At4CL2<-pPDC1-pTPI1->VvVST1)-DR

Plasmopara viticola strains

Plasmopara viticola spores were collected in the vineyards of the Dienstleistungszentrum Ländlicher Raum Rheinpfalz (Neustadt an der Weinstraße, Germany) and propagated on freshly collected *V. vinifera* cv. 'Müller Thurgau' leaves. The leaves were sprayed with a sporangia suspension in sterile

water (ca. 45,000 per ml) on the abaxial leaf surface. The wet chambers were sealed and incubated overnight at 25 °C. After this incubation period, the remaining water droplets were removed and the leaves at the same conditions for 6 days. Leaves were selected for sporangiophores on the inoculated surface and directly used for production of a sporangia suspension for the experiments.

Production of competent bacteria and agrobacteria strains

Bacteria were made chemically competent with the Inoue-Method as described by Inoue and colleagues 1990 (Inoue et al., 1990).

Agrobacteria were made electrically competent. A single colony was inoculated in 2x 25 ml YEB-medium (Rif15) and incubated over night at 25 °C and 120 rpm to an OD_{600} 0.5-0.6. At this OD, bacteria were put on ice for 30 min and then centrifuged in a pre-cooled centrifuge for 10 min at 5,000 rpm. The supernatant was discarded, the pellets were resuspended in 50 ml ice-cold H_2O. This process was repeated once but at 6,000 rpm, then again but with resuspension in 25 ml 10 % (v/v) glycerol and last time, at 5,000 rpm and 400 µl 10 % (v/v) glycerol. All steps were carried out with ice cold media. 100 µl of the cell suspension were aliquoted into pre-cooled 1.5 ml Eppendorf tubes, frozen in liquid nitrogen and stored at -80 °C.

4.4 Media and antibiotics

Grapevine cell suspension culture medium

For the grapevine cell suspension culture, Grape Cormier (GC) medium was adapted from (Bao Do and Cormier, 1991): 3.2 g/l Gamborg B5 with minimal organics (Sigma medium G5893), 30 g/l sucrose, 0.25 g/l casein hydrolysate, 0.93 g/l kinetin, 0.54 g/l NAA, pH5.8

Table 6: Antibiotics

Antibiotic	Solvent	Final concentration [g/ml]
Carbenicillin (replacement for Ampicillin)	50 % (w/v) Ethanol	50
Kanamycin	Water	50
Rifampicin	Methanol	50
Spectinomycin	Water	50

LB

10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl, (for solid medium: 15 g/l agar).

SOC medium

20 g/l tryptone, 5 g/l yeast extract, 0.5 g/l NaCl

2YT

16 g/l tryptone, 10 g/l yeast extract, 5 g/l NaCl, (for solid medium: 15 g/l agar).

YEB

5 g/l beef extract, 5 g/l peptone, 1 g/l yeast extract, 5 g/l sucrose, 0.5 g/l $MgSO_4$ (pH 7.5) (for solid medium: 15 g/l agar).

YPD-Medium

20 g/l peptone, 10 g/l yeast extract, 20 g/l glucose (for solid medium: 20 g/l agar).

Table 7: Yeast selection media (Amino acid dropout)

Name	GAL-medium (1 l)*	GLC-medium (1 l)*
Yeast-nitrogen-base w/o aminoacids	7 g	7 g
Aminoacid dropout**	1,3 g	1,3 g
Raffinose	10 g (1 % (w/v))	20 g (2 % (w/v))
Galactose	10 g (1 % (w/v))	0 g
Glucose	0 g	1 g (0,1 % (w/v))
Bacto-agar (2 % (w/v)) (for solid medium)	20 g	20 g

* GAL is the inducing galactose medium, GLC is the inhibiting glucose medium.

** Amino acid dropout contains:

+L-Ade, +L-Arg, +L-Asp, +L-Asn, +L-His, +L-Ile, +L-Lys, +L-Met, +L-Phe, +L-Pro, +L-Ser, +L-Thr, +L-Trp, +L-Tyr and +L-Val in equal concentration. Depending on the vector either +L-Leu or +L-Ura are added and the other left out for selection.

Glycerol stocks

Bacterial strains that were prepared for storage of plasmid or the respective empty strain were grown in 5 ml LB medium with the appropriate antibiotic at 37 °C and 180 rpm overnight. 400 µl were pipetted into a sterile screw-cap tube (2 ml), mixed with 600 µl 50 % glycerol (sterile), shock-frozen in liquid nitrogen and stored at -80 °C.

4.5 Nucleic acid methods

Oligonucleotides

Table 8: Oligonucleotides for cloning

Oligonucleotid name	Sequence (5'-3')
STS29pF	TATGAGCTCAAAATGTCGAAACACTTTGTATTAAA
STS29pR	TATCTCGAGATGCCAGATACGTTCTGAAATTG
GT_VIT02_01240_attB1-F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCAAGCCCAAAACGAAGATCCTT
GT_VIT02_01240_attB2-R	GGGGACCACTTTGTACAAGAAAGCTGGGTATTCCATGGTGAATAAACCGAG
StLACpro_attB1-F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCTTACCAATTTACAAAACAGACA
StLAcpro_attB2-R	GGGGACCACTTTGTACAAGAAAGCTGGGTCATGATCAGCCACATCTTCTTCGGAA
ABC12_VIT_09s0002g05570_attB1_F1	GGGGACAAGTTTGTACAAAAAAGCAGGCTAAATGTATGTATTTGAAATTAT
ABC12_VIT_09s0002g05570_attB1_R2	GGGGACCACTTTGTACAAGAAAGCTGGGTATTGCAACACGAAACGAGGAAC
ABC13pro_VIT_07s0005g02660_attB1_F1	GGGGACAAGTTTGTACAAAAAAGCAGGCTTTCATCACCATGTAGATAATCTT
ABC13pro_VIT_07s0005g02660_attB1_F2	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATTTTTTAGGTAAAATTTTTA
VvGLUC1proF1_attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCAGAAGTTAATATTAACATCGATG
VvGLUC1proR_attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTCCATATTGGAGAATATTCAAAGGA

Oligonucleotid name	Sequence (5'-3')
VvGLUC2proF1_attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGATAAAGCAAGCTCCAATCCAAC
VvGLUC2proR_attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTCTGAAGAACTTCTACTAGTTCTAAG
MYB15_GW_F	GGGGACAAGTTTGTACAAAAAAGCAGGCTCGATGGTAAGAGCTCCTTGT
MYB15_GW_R	GGGGACCACTTTGTACAAGAAAGTGGGTCTCAAAGCTCCTGTAAGCC
VvMYB14-hp1-attB1-F	GGGACAAGTTTGTACAAAAAAGCAGGCTAGACACTCTCTTGATGCGTC
VvMYB14-hp1-attB2-R	GGGGACCACTTTGTACAAGAAAGCTGGGTCCTCATATTTCTGATAATTCATGC
MYBA1prof	ATAGAGCTCTTTTTAACCCGCCCATTTTT
MYBA1proR	ATAGTCGACCCTTTCTAACTCCTAAGCTCTCCATC
MYBA2proF	ATAGAGCTCGGTGTGCAAAATCATAACTTTCTTCT
MYBA2proR	ATAGTCGACCCCTTTCTAACTCCTAAGCTCTTCATC
VvGT2_GWA	GGGGACAAGTTTGTACAAAAAAGCAGGCTTC GAAGGAGATAGAACCATGGGGTCTGAATCAAAGCTA
VvGT4_GWA	GGGGACAAGTTTGTACAAAAAAGCAGGCTTC GAAGGAGATAGAACCATGGAACACCCAACACCTC
VvGT5_GWA	GGGGACAAGTTTGTACAAAAAAGCAGGCTTC GAAGGAGATAGAACCATGATGAAGAAAATGGAGCTTATTT
VvLAC_GWA	GGGGACAAGTTTGTACAAAAAAGCAGGCTTC GAAGGAGATAGAACCATGTGGCTGATGATGAAGGTTT
VvGT2_GWC	GGGGACAAGTTTGTACAAAAAAGCAGGCTTC ATGGGGTCTGAATCAAAGCTA
VvGT4_GWC	GGGGACAAGTTTGTACAAAAAAGCAGGCTTC ATGGAACACCCAACACCTC
VvGT5_GWC	GGGGACAAGTTTGTACAAAAAAGCAGGCTTC ATGATGAAGAAAATGGAGCTTATTT
VvLAC_GWC	GGGGACAAGTTTGTACAAAAAAGCAGGCTTC ATGTGGCTGATGATGAAGGTTT

Oligonucleotid name	Sequence (5'-3')
VvGT2_GWL	GGGGACCACTTTGTACAAGAAAGCTGGGTG AATTTTCTTTGACTTGCAAACCA
VvGT4_GWL	GGGGACCACTTTGTACAAGAAAGCTGGGTG AACCTTGATATTCTTCCATATCT
VvGT5_GWL	GGGGACCACTTTGTACAAGAAAGCTGGGTG TGAGATATTAGTTATCATGTCTCC
VvLAC_GWL	GGGGACCACTTTGTACAAGAAAGCTGGGTG ACATGGTGGCATGTCGGAT

Table 9: Oligonucleotids for sequencing

Primer name	Sequence 5'-3'	Plasmid (direction)
SeqLA	TCGCGTTAACGCTAGCATGGATCTC	pDONR201 (fw)
SeqLB	GTAACATCAGAGATTTTGAGACAC	pDONR201 (rv)
M13forward	GTAAAACGACGGCCAGT	pDONR221 (fw)
M13reverse	GGAAACAGCTATGACCATG	pDONR221 (rv)
35S	CAATCCCACTATCCTTCGCAA	pART7 + pART27 + pB7WG2D + pEG203 (fw)
OCSrev	GGCGGTAAGGGAGCTA	pART7 + pART27 + pEG203 (rv)
LUCF	CTAACATACGCTCTCCATCA	pLUC (fw)
LUCR	GGATAGAATGGCGCCGG	pLUC (rv)
NusA_fw2	CTGATTATGGCTGCCCGT	pHNGWA (fw)
T7term	CTAGTTATTGCTCAGCGGT	pHNGWA + pHGGWA + pETG-vectors (rv)
GT2 rev	AGA TGG CTC TCC TCA GGA AT	VvGT2 in pHNGWA, 5' section (rv)
GST_fw2	ATATAGCATGGCCTTTGCAG	pHGGWA (fw)
T7prom	TAATACGACTCACTATAGGG	pETG-vectors + pESC-GWA vectors (fw)
Myc-tag rv	TCGGAAATCAACTTCTGTTC	pESC-GWA (rv)

Primer name	Sequence 5‘-3‘	Plasmid (direction)
Do15_rev	CTGTTCGAGGTCCTCCTCTG	GreenGate (GG) cloning system, D-module (rv)
B21_fw	AAGGGCGAGGAGGATAACAT	GG, B-module (fw)
A00111	ACCTCTCGGGCTTCTGG	GG, LB region (fw)
A01924	CGATTTTTGTGATGCTCGTC	GG, RB region (rv)

Oligonucleotids were ordered either at Eurofins Genomics (Luxembourg) or at Biomers (Ulm, Germany) in cartridge-purity and lyophilized.

Plasmids

Table 10: List of used plasmids

Plasmid name	Function	Resistance	Reference
pART7_GW	Gateway compatible cloning vector (for pART27)	Amp	Gleave (1992) Poschet (2012)
pART27	Binary vector for plant transformation, 35S promoter	Amp	Gleave (1992)
pB7WG2D	Binary vector for plant transformation, 35S promoter, ER-GFP	Spec / Basta	Karimi et al. (2007)
pDONR201	Entry vector for the Gateway cloning system	Kan	Invitrogen
pDONR221	Entry vector for the Gateway cloning system	Kan	Invitrogen (#12536017)
pEarleyGate203	Plant transformation vector, gateway compatible, N-terminal Myc-tag	Kan	Earley et al. (2006)
pESC-LEU	Expression vector for yeast, GAL inducible, LEU2 selection marker	Amino acid selection	Agilent technologies Catalog #217452

Plasmid name	Function	Resistance	Reference
pESC-URA	Expression vector for yeast, GAL inducible, URA3 selection marker	Amino acid selection	Agilent technologies Catalog #217454
pETG-10A	Expression vector, IPTG inducible, His-tag	Amp	A. Geerlof, EMBL
pETG-30A	Expression vector, IPTG inducible, His-tag, N-GST-tag	Amp	A. Geerlof, EMBL
pHGGWA	Expression vector, IPTG inducible, His6-GST	Amp	Busso et al. (2005)
pHNGWA	Expression vector, IPTG inducible, His6-NusA	Amp	Busso et al. (2005)
pLUC_GW	Gateway compatible vector for promoter – luciferase fusion	Amp	Horstmann et al. (2004) Poschet (2012)
pGGA006	GreenGate module A006	Amp	Lampropoulos et al. (2013)
pGGB21	GreenGate module B21	Amp	Lampropoulos et al. (2013)
pGGC087	GreenGate module C087	Amp	Lampropoulos et al. (2013)
pGGD015	GreenGate module D015	Amp	Lampropoulos et al. (2013)
pGGE001	GreenGate module E001	Amp	Lampropoulos et al. (2013)
pGGF005	GreenGate module F005	Amp	Lampropoulos et al. (2013)
pGGZ003	GreenGate module Z003	Spec	Lampropoulos et al. (2013)

Cloning and cloning systems

The cloning of promoters and open reading frames (ORFs) was performed with the Gateway® technology with clonase II by Invitrogen (Catalog nos. 12535-029 and 12535-037).
To amplify the desired DNA sections, primers were designed according to the instructions of the manual (attB overhangs are necessary), see also primer tables. The Polymerase chain reaction (PCR) was done with a Phusion polymerase (Thermo Fisher) mainly for candidate gene cloning or a Taq DNA-polymerase (Sigma-Aldrich, D1806) mainly for promoter and transcription factor cloning. The PCRs were performed according to the respective manufacturers manuals in a cycler, using a temperature gradient for the annealing temperature of 10 °C around the calculated primer optimum for better results. In especially difficult cases, for example GC-rich regions, a PCR enhancer was used in addition (Ralser et al., 2006). Checks of the results were either done by restriction enzyme digestion (Fast-digest enzymes by Thermo Fisher, according to manual), check PCR (Taq DNA-polymerase, Sigma-Aldrich) or sequencing (Eurofins genomics). For promoter cloning, gDNA, provided by Dr. Janine Höll was used (Höll, 2014). For cloning of the ORFs, cDNA from *V. vinifera* cv. 'Pinot Noir' was used. The material was mixed from infected leaf tissue, grape berries and root tissue in order to include material with suitable gene expression for all possible genes. After size check on an agarose gel, the PCR product was purified as suggested and described in the Gateway manual. The BP and LR reactions and all associated steps were carried out as described by the manufacturer. *E. coli* TOP10 cells were used for plasmid propagation in between the steps, as well as for long term storage at -80 °C. *E. coli* DB3.1 cells were used for ccdB containing plasmids. As entry vectors either pDONR201 or pDONR221 were used. The destination vectors depended on the experiment, see plasmid list.

Cloning of the MYB transcription factors for stable grapevine transformation was started and mainly conducted by Dr. Janine Höll and then continued and re-tested by me. The instructions in the publication (Lampropoulos et al., 2013) as well as personal communication with the developing AG Lohmann (COS, Uni Heidelberg) were followed. For an overview of the used modules, see plasmid table.

DNA agarose gel electrophoresis

For analysis by agarose gel electrophoresis, DNA samples were mixed with 6x DNA Gel loading dye (Thermo Fisher, R0611) and run on a 1 % (w/v) agarose gel with 3 µl/100 ml Midori-green DNA stain (Nippon genetics, Germany) in TAE buffer at 100 V for 30-45 min. The "Thermo Scientific GeneRuler 1 kb DNA Ladder" (SM0311) was used as molecular weight marker.

Transformation procedures of bacteria, agrobacteria and yeast strains

The transformation of plasmids into *E .coli* was performed with a slightly modified Gateway protocol. The heat shock time was increased to 45 sec and the amount of SOC medium increased to 1 ml. Furthermore, the bacteria suspension in SOC was not diluted before plating but taken directly (plasmid re-trafo) or increased in concentration by careful centrifugation (4,000 g, 5 min, for BP/LR-reaction products).

S. cerevisiae transformation was carried out with a protocol adapted from a previous publication (Gietz and Schiestl, 2007). Pre cultures of EFSC3672 and EFSC3497 strains were picked from a single colony, transferred into 5 ml YPD medium and incubated in a 15 ml reaction tube at 30 °C and 200 rpm overnight. From the pre cultures, 200 µl were used to start a main culture of 5 ml, which was grown under the same conditions until it reached an OD_{600} of 0.5. The culture was then centrifuged for 5 min at 3,000 g in a swing bucket rotor. The cells were then washed once in the same amount of sterile water and centrifuged again. Meanwhile, single stranded carrier DNA was boiled for 5 min and then kept on ice. The cells were resuspended in 1 ml LiAc (100 mM) and transferred into a 1.5 ml reaction tube. They were then centrifuged at 2,500 g for 5 min and resuspended in 50 µl LiAc (100 mM). The mixture was then incubated for 30 min at room temperature, after which 5 µl of the denatured carrier DNA combined with 0.1 µg DNA (Plasmid, in 50 µl) were added to the cells. 300µl PEG/LiAc/TE-buffer mix (40 % (w/v) polyethylene glycerol 3350 in 100 mM LiAc, solved in TE-buffer) were added. The cells were then incubated at 30°C for 30 min and subsequently heat shocked for 15 min at 42 °C. The cells were then centrifuged at 3000g for 3 min, the supernatant discarded, the cells resuspended in 100 µl ddH_2O and plated on YPD plates, which were incubated at 30 °C overnight.

Agrobacteria were transformed with the electroporation method. 1 µl of DNA (100-200 ng) were pipetted into the 100 µl aliquot from competent agrobacteria production and kept on ice for 20 min. The electrical shock of 2.2 V was applied in sterile metal cuvettes (0.1 cm) after which the cells were instantly transferred into 0.5 ml 2YT medium and incubated gently shaking at 28 °C for 1 h. After this, the cells were plated on 2YT plates with the appropriate antibiotics.

Plasmid minipreps

Plasmids were extracted from bacterial liquid cultures with the GeneJET Plasmid Miniprep Kit (Thermo Fisher, K0502) according to manufacturer's instructions. The concentration was subsequently measured with a NanoDrop 2000 (Thermo Fisher).

RNA extraction and gDNA digestion

RNA extraction was done with two methods, depending on the tissue.

For berry tissue, a hot borate RNA preparation protocol was used, since the high metabolite content led to very low, if any, yield. In this protocol, 100 mg ground plant material, cooled in liquid nitrogen was mixed with 700 µl Hot Borate buffer (0.2 M sodium tetraborate decahydrate (Sigma S9640), 30 mM EGTA (Sigma E4378), 1 % (w/v) SDS, 1 % (w/v) deoxicholate sodium (Sigma D6750) to pH 9 and then freshly mixed with 0.2 µl antifoam A (Sigma A6582), 14 mg polyvinylpyrrolidone (Serva 33422), 1.08 mg DL dithiothreitol (Sigma D0632) per 700µl buffer) that was pre-heated to 80 °C. After vortexing for 30 sec, 15 µl proteinase K (10mg/ml) was added and the sample was gently shaken at 42 °C for 90 min. Then 56 µl 2 M KCl were added and the sample was incubated on ice for one h. After 20 min of centrifugation at 12000 g and 4 °C, 250 µl 8 M LiCl was added and the sample was incubated on ice in the fridge overnight. The next day, the sample was centrifuged as before, the supernatant was discarded and the pellet resuspended in 500 µl ice-cold 2 M LiCl. This was repeated once exactly the same and then with 200 µl 10 mM Tris-HCl for resuspension (ice cold). Afterwards, 20 µl 2 M KAc (pH 5.5) was added and the sample incubated on ice for 15 min. After centrifugation for 10 min at 12,000 g and 4 °C, the supernatant was transferred into a fresh reaction tube, mixed with twice the volume of ice-cold pure ethanol and incubated at -80°C for 2 h. Then the sample was centrifuged for 30 min at 12,000g and 4°C, the supernatant was discarded and the pellet washed with 1 ml ice cold 70 % (v/v) ethanol. After another centrifugation for 15 min, the supernatant was discarded, the pellet air dried, resuspended in 50 µl TE-buffer and the concentration measured with a NanoDrop 2000 (Thermo Fisher). Digestion of gDNA was performed with a commercially available kit (Sigma Aldrich).

For all tissues except berries, the "Spectrum™ Plant Total RNA-Kit" (Sigma Aldrich, STRN250) with the additional on-column DNase I digestion set (Sigma Aldrich, DNASE70-1SET) was used according to manufacturer's instructions. Concentrations were measured with a NanoDrop 2000 (Thermo Fisher).

cDNA

For cDNA synthesis from the extracted RNA, 500 ng RNA was mixed with 1 µl oligo dT (10 µM), 1 µl dNTPs (10 mM) and filled up to 14 µl with sterile H_2O. The mix was then incubated at 65 °C for 5 min and transferred back on ice for the addition of 4 µl reverse transcriptase buffer (5x), 1 µl DTT (100 mM), 0.5 µl RNAse Inhibitor and 0.5 µl AMV reverse transcriptase (all in a 6 µl mix). The total mix was incubated for 20 min at 42 °C and 45 min at 50 °C before finally 30 ml sterile H_2O were added and the mix was stored at -20 °C until further use. Before use in qRT-PCR, an aliquot was taken and diluted 1:10 with sterile H_2O.

4.6 Microscopy

The new inducible constructs of VvMYB14, VvMYB15 and an empty control were analyzed in *N. benthamiana* for their mCherry signal. Confocal laser scanning microscopy images were taken with the help of R.Röhrich (COS Heidelberg, Schumacher laboratory) using a Leica TCS SP5II microscope with a Leica HCX PL APO lambda blue 63x 1.20 UV water immersion objective. mCherry was excited at 561 nm with a VIS-DPSS 561 laser diode and emission was detected at 600-650 nm. Background fluorescence emission was detected at 700-750 nm.
For positive control, a VHA-a1-mCherry construct by Dr. Upendo Lupanga (COS Heidelberg, Schumacher laboratory) was used (Lupanga et al., 2020).

4.7 Infection of leaf discs

The infection of *V. vinifera* leaf discs with *P. viticola*, the two cultivars 'Regent' (resistant, Rpv 3.1) and 'Pinot Noir' (susceptible) were chosen. The experiments were performed in cooperation with Chantal Wingerter. The fourth and fifth leaves below the shoot apex were chosen and leaf discs of 1.5 cm diameter were excised with a cork borer. They were then put onto wet, sterile filter papers placed in a petri dish. The *P. viticola* sporangia suspension (see above, 10 µl containing 45,000 sporangia per ml) or sterile dH_2O as negative control were applied to the abaxial leaf surface. The leaf discs were incubated at 22 °C for twelve h after which the remaining water drops on the leaf surface were removed. The petri dishes were then sealed and incubated at the same temperature with a light-dark cycle of 16 h light and 8 h dark. Samples were taken 0, 6, 8, 12, 24 and 48 h after infection. Per treatment (infected or mock) and per cultivar ('Regent' and 'Pinot Noir'), five plants were used. Of each plant, two leaf discs were pooled with the ones from the remaining four corresponding plants. Before freezing in liquid nitrogen, the outer edge of each leaf disc was removed by cutting with a 13 mm cork borer. This is supposed to reduce the impact of mechanical wounding on the results.

4.8 Quantitative real time PCR

Table 11: qPCR primers

Gene	Sequence (5'-3')	Reference
VvMYB14 (fw)	TCT GAG GCC GGA TAT CAA AC	Höll et al (2013)
VvMYB14 (rv)	GGG ACG CAT CAA GAG AGT GT	Höll et al (2013)
VvMYB15 (fw)	CAA GAA TGA ACA GAT GGA GGA G	Höll et al (2013)
VvMYB15 (rv)	TCT GCG ACT GCT GGG AAA	Höll et al (2013)
VvSTS25/27/29 (fw)	TGT CAA GTG CAT GTG TGT TG	Höll et al (2013)
VvSTS25/27/29 (rv)	AGT CAA GCC TGG TCC AAA AC	Höll et al (2013)
VvGT2 (fw)	TTC AGG AGT TTG TGG ACG AG	Designed for the project
VvGT2 (rv)	ACC CTA TGA TCA TGA GGA CGT	Designed for the project
VvGT4 (fw)	CTG CGA CAA TGG CTC TGA G	Designed for the project
VvGT4 (rv)	TCC AAT TCT TCC ATT TCA ACC CT	Designed for the project
VvGT5 (fw)	TTG ATG GAG GAT AGC AAT GAC A	Designed for the project
VvGT5 (rv)	TCT CCA ATT AGA CGT TGT AAG CT	Designed for the project
VvLAC (fw)	CAC GGC TCG CTA CAA GTA TAA	Designed for the project
VvLAC (rv)	GTG GTG GAT GGC TAG GAA ATT	Designed for the project
VvABC12 (fw)	AGT GGT TGC AGC TGT GAT TG	Designed for the project
VvABC12 (rv)	CTC GTG CTT TTA CCG CCT TT	Designed for the project
VvABC13 (fw)	AGG GAA AGC ATG AAA CTC TGA	Designed for the project
VvABC13 (rv)	GGG CAT CAC AAC AAG GCT TT	Designed for the project
VvPPO (fw)	AGCCGAAGATGATGAGAGTG	Designed for the project
VvPPO (rv)	ATCATTGAAAGGTGGGATCA	Designed for the project
VvROMT (fw)	TGCCTCTAGGCTCCTTCTAA	Schmidlin et al. (2008)
VvROMT (rv)	TTTGAAACCAAGCACTCAGA	Schmidlin et al. (2008)
VvUbi (fw)	GTG GTA TTA TTG AGC CAT CCT T	Reid et al. (2006)
VvUbi (rv)	AAC CTC CAA TCC AGT CAT CTA C	Reid et al. (2006)
VvGAPDH (fw)	CCA CAG ACT TCA TCG GTG ACA	Reid et al. (2006)
VvGAPDH (rv)	TTC TCG TTG AGG GCT ATT CCA	Reid et al. (2006)
VvEF1a (fw)	AAC CAA AAT ATC CGG AGT AAA AGA	Reid et al. (2006)
VvEF1a (rv)	GAA CTG GGT GCT TGA TAG GC	Reid et al. (2006)

Procedure

Quantitative real time PCR (qPCR) was performed on a Rotor-Gene Q (Quiagen, Germany). The reaction mix was based on the Maxima™ SYBR™ Green 2x qPCR Master Mix (Thermo Scientific, K0252) but modified. One reaction included 10 µl Master mix, 0.6 µl of each, forward and reverse primer (10 µM stock concentration), 2.64 µl of the 1:10 diluted cDNA template (see cDNA synthesis) and 6.16 µl sterile H_2O. The cycling program was 95 °C for 10 min, then 40 cycles of 95 °C for 15 sec, 58 °C for 30 sec, and 72 °C for 20 sec. Finally, a melt cycle was run with 1 °C increments in 5 sec steps from 56 to 96 °C. Initially, the primer efficiency was tested in dilution series of cDNA from *V. vinifera* cv. 'Pinot Noir' tissue from grape berries of all development stages and leaf tissue (infected and uninfected) in a mix. An efficiency between 90 % and 105 % was accepted.

For normalization of the cycle threshold (CT) values, the reference genes *VvUbiquitin1* (TC32075), *VvEF1-α* (EC959059) and *VvGAPDH* (CB973647) were used since they were already established as reliable reference genes during berry development (Reid et al., 2006). Determination of CT values and analysis of melting curves was done with the Rotor-Gene Q Series Software Q (QIAGEN GmbH Germany) 2.0.2 and the Q-Gene software (Muller et al., 2002). Analysis of results and normalization against these reference genes was conducted according to the ΔΔCT method (Pfaffl, 2001).

4.9 HPLC

Stilbene extraction, various tissues and cultures

For stilbene extraction from *V. vinifera* and *N. benthamiana* tissue, 50 mg of the frozen material were ground to fine powder with a Mixer Mill MM400 (Retsch, Germany) at 30 Hz for 30 to 45 sec. 400 µl of 80 % (v/v) methanol (HPLC grade, Merck, Germany) were added and the samples were vortexed for 30 sec. For the yeast cultures (induced and uninduced), 100 µl were mixed with 400 µl methanol (HPLC grade), vortexed for 30 sec. After sonication for 20 min in an ice-water bath, both sample types then were centrifuged for 15 min at 13,000 rpm and 4 °C. The supernatant was transferred into a fresh 1.5 ml reaction tube and evaporated using a concentrator plus (Eppendorf, Germany) at 60 °C. The samples were then resolved in 50 µl of 50 % (v/v) methanol (HPLC grade), diluted with 200 µl H_2O (HPLC grade) and after centrifugation as before for 15 min, the supernatant was filled into glass HPLC vials and analyzed.

Standards

Standards of *trans*-resveratrol, *trans*-piceid, *trans*-pterostilbene, ε-viniferin, *trans*-oxy-resveratrol and *trans*- 2,3,4',5-tetrahydroxystilben-2-glycosid were purchased from Phytolab (Germany). They were mixed and diluted to 0.5, 1, 5, 10 and 25 pmol/µl mixes, which were measured in a dilution series on the HPLC for identification and quantification of the stilbene compounds in our samples.

Measurement

The extracted stilbenes were analyzed via HPLC (Jasco 4000er, Jasco AS-4150 autosampler, Jasco PU-4180 pump, Jasco MD-4010 PDA). The samples were separated on a reverse phase column (Gemini-NX 3 µm, C18 110 Å, LC column 150 x 4,6 (Phenomenex ® LTD, Aschaffenburg, Germany) with mounted pre-column) with a gradient of buffer A (10 mmol KH_2PO_4; 5 % (v/v) acetonitrile (HPLC grade); 95 % (v/v) water (HPLC grade); pH adjusted with 85 % (v/v) $H3PO_4$ to 1.5) to buffer B (10 mmol KH_2PO_4; 50 % (v/v) acetonitrile (HPLC grade); 50 % (v/v) water (HPLC grade); pH adjusted with 85 % H_3PO_4 to 1.5). The gradient conditions were as follows: 0 min, 90 % A; 7 min, 66 % A; 12 min, 49 % A; 17 min, 32 % A; 22 min, 0 % A; 28 min, adjustment back to 90% A. The flow rate was 1 ml per min and the column was kept at 25 °C. The stilbenes were measured at 320 nm excitation wavelength by the photo diode array detector. The emission detector was set to a wavelength range of 200-650 nm. ChromNav Software provided by Jasco was used for data acquisition and analysis.

4.10 Promoter induction assays

Particle gun DNA bombardement

For the transient promoter assays, the cell suspension culture of *V. vinifera* cv. 'Chardonnay' that was described above, was used with a modified protocol based on an earlier publication of the work group (Czemmel et al., 2009; Höll, 2014). 1.6 µm gold particles (Bio-Rad) were coated with 2 µg plasmid, made up of 0.5 µg of each separate plasmid or empty vectors if the total amount was not reached. pLUC, a plasmid carrying renilla luciferase was used as internal control as described before (Horstmann et al., 2004). The plasmid coated particles were then shot into the cells with the PDS-1000/He Biolistic Particle Delivery System by Bio-Rad with 4481 kPa helium pressure, a vacuum of 86 kPa and a distance of 9.5 cm. The cells were then incubated on GC medium for 48 h at 22 °C in darkness. They were harvested by grinding with a mortar on ice cold equipment with addition of 200 µl of 2x Passive Lysis Buffer (Promega). The samples were centrifuged for 1 min at 10,000 rpm. For the measurement of the

luciferase activity, the dual-luciferase reporter assay system by Promega was used. 10 µl of the sample's supernatant was mixed with 25 µl LARII and Stop & Glo®. The luciferase light emission measurement was performed with a Lumat LB 9507 Luminometer (Berthold Technologies). The ratio between emission of the firefly luciferase and the renilla luciferase was calculated and background luminescence of untransformed cells was subtracted.

4.11 Protein expression + Enzymatic assay UPLC-MS

Expression and purification

The bacterial expression of *VvGT2* and *VvGT5* was done in *E. coli* cells of the BL21 (DE3) strain. From a single colony, a pre culture was grown in 3 ml LB medium with the appropriate antibiotic in 10 ml culture tubes, shaking at 170 rpm and 37 °C over night. A main culture was then started with 250 µl of the pre culture in 25 ml LB medium with the appropriate antibiotic and grown for three h at the same conditions (OD_{600} 0.7-0.8). Induction was done by adding IPTG to a final concentration of 1 mM and incubation at 37 °C and 170 rpm for 4 h (separating some uninduced culture as negative control under the same conditions). The samples were then centrifuged at 4,500 g and 4 °C for 10 min. The pellet was then either frozen for later use or used directly.

SDS PAGE

The induced and uninduced samples (pellet of 1 ml) was resolved in 200 µl PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4) buffer, sonified for 3x 30 sec and centrifuged again. 100 µl were taken, mixed with 300 µl methanol, 100 µl chloroform was added and the sample vortexed. 300 µl sterile H_2O was added, again vortexed and centrifuged at 12,000 rpm at room temperature for 2 min. The upper phase was removed, 300 µl methanol added and the sample vortexed and centrifuged as before. The supernatant was discarded, the pellet air dried and resuspended in 20 µl sterile H_2O and 20 µl (2x) Laemmli buffer (4 % (w/v) SDS, 20 % glycerol, 120 mM tris-HCl and 0.02 % (w/v) bromphenol blue). The sample was then mixed again and loaded on the polyacrylamid gel.
The PAA gels were freshly prepared. The 12 % resolving gel was prepared with 1.75 ml resolving gel buffer (1.5 M tris base, 0.4 % (w/v) SDS, pH 8.8), 2.8 ml acrylamide, 2.45 ml ddH_2O, 7 µl TEMED and 30 µl 10 % APS. The stacking gel was made from 1.25 ml stacking gel buffer (0.5 M Tris base, 0.4 % (w/v) SDS, pH6.9), 0.75 ml acrylamide, 3 ml ddH_2O, 8 µl TEMED, 60 µl 10 % (v/v) APS and 15 µl coomassie staining solution. All chemicals for the PAA gels were ordered from Carl Roth (Germany).

The gels were prepared and run in equipment of the "Mini-PROTEAN Tetra Vertical Electrophoresis Cell" series by Biorad (USA). The running buffer contained 25 mM tris base, 192 mM Glycin, 0.1 % (w/v) SDS at pH 8.3. The samples were run at 120 V for 15 min through the stacking gel and at 200 V for about 1 h through the resolving gel. The PAA gels were stained for 10 min in 10 % acidic acid and 0.2 % Coomassie R250 and then destained in 10 % acidic acid overnight.

Protein extraction

Frozen pellets of 20 ml culture were resuspended in 4 ml buffer (50 mM hepes (pH 7), 5 mM $MgCl_2$, 14 mM β-mercaptoethanol) and sonicated in 50 ml reaction tubes, at level 2-3 of 10 (no foaming) for 3x10 sec on ice. 100 µl as crude extract (CR) fraction. After centrifugation for 5 min at 12,000 rpm, 100 µl were taken as supernatant (SN) fraction.

Enzyme assay

The enzymatic assays were performed with 100 ml crude extract from above, 2 µl UDP-glucose (Sigma Aldrich), 2 µl substrate in a total volume of 200 µl (filled up with buffer as above). The samples were incubated overnight at 28 °C. The next morning, the samples were heated to 80 °C for 5 min, 200 µl acetone was added and centrifuged at 13,000 rpm for 10 min. 100 µl of the supernatant was filled into polypropylene vials and analyzed. The tested substrates were: 3,5-dihydroxyanisole (DHA), 3-phenylphenol, 4-phenylphenol, 4-vinylphenol, ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)), caffeic acid, catechin, chlorogenic acid, citronellol, esculetin, ethyl gallate, eugenol, farnesol, gallic acid monohydrate, geraniol, guaiacol, homovanillic acid, linalool, myricetin, naringenin, nerolidol, olivetol, phenethyl alcohol, quercetin, resveratrol and terpineol. They were added from 1mM stock solutions in methanol, either as mix or separate, as needed.

UPLC-MS measurement

UPLC-MS analysis wasa conducted as described before by the group of Dr. Philippe Hugueney (MSV, INRAE Grand Est, Colmar) with three alterations (Koutouan et al., 2018).
The column was kept at 20°C instead of 30°C. The gradient was set differently: 0–1 min, 95 % B; 1–2 min, 95–80 % B; 2–3.5 min, 80–5 % B; 3.5–4.4 min, 5 % B; 4.4–5 min 5–95 % B; 5–5.5 min 95 % B. And finally, the spectra were acquired within the mass-to-charge ratio (m/z) range of 95–1000 atomic mass units (a.m.u.).

4.12 Expression in yeast strains

The transformed *S. cerevisiae* cells of the original strains EFSC3672 and EFSC3497 were selected by plating them on selection plates lacking uracil or leucine, respectively. Positive colonies were chosen and transferred to a 5 ml liquid culture of the same selection medium (glucose based to prevent induction) and incubated shaking at 30 °C, 200 rpm and in darkness overnight. From these pre cultures, fresh main cultures in 100 ml selection medium were prepared, with one batch being induced in a galactose medium version and the other batch as negative control in galactose medium. Growth conditions as described. OD_{600} density measurements were taken regularly and after 2, 4, 6 and 24 h, 1 ml sample was taken, frozen in liquid nitrogen and stored at -80 °C (dark) until stilbene extraction.

4.13 Infiltration of *N. benthamiana* leaves

Infiltration

Agrobacteria were grown from a single colony in 10 ml LB medium with the appropriate antibiotics overnight in a 100 ml Erlenmeyer flask. The cells were then centrifuged at 4500 rpm and 4 °C for 15 min. The pellet was then resuspended in 2 ml infiltration solution (10 mM MES-KOH, pH 5.6, 10 mM $MgCl_2$ and 150 µM acetosyringone). The OD_{600} was measured and set to 0.8 with infiltration solution. The mixture was incubated for 2-3 h at room temperature. Tobacco plants were watered in the morning and the bacteria mixture was pressed into the leaves from the abaxial side through 10 ml syringes without needle. The plants were then kept in the greenhouse for 2-5 more days before harvesting and analysis.

4.14 *In silico* methods

Chemical structures were drawn using the MarvinScetch 20.21 software by ChemAxon (Hungary).
For analysis of experimental results and the respective graphs, Excel 2019 (Microsoft) and GraphPad PRISM 8 was used.
Subcellular localization was predicted using the WoLF PSORT protein localization predictor (Horton et al., 2007).
Fiji (Schindelin et al., 2012) was used for adding the scale bar into the microscopy images.

Phylogenetic analysis

The protein sequences were chosen from related publications and/or NCBI genbank annotations. The accession numbers can be found below. Alignment of protein sequences and phylogenetic analysis was carried out using the MEGA-X program (Kumar et al., 2018). The sequences were aligned using ClustalW (Chenna et al., 2003). Evolutionary relationship was calculated using the Neighbor-Joining method (SAITOU 1987) and tested using the bootstrap method (Felsenstein, 1985) with 10000 replicates. Therein the evolutionary distances were computed using the Poisson correction method (Zuckerkandl and Pauling, 1965). The units are the number of amino acid substitutions per site.

Table 12: Proteins analyzed in the phylogenetic analysis, their accession numbers and publications (if not direct NCBI submissions).

The species abbreviations are as follows: At = *Arabidopsis thaliana*; Bc = *Botrytis cinerea*; Br = *Brassica rapa*, Gv = *Gypsophila vaccaria*; Hs = *Homo sapiens*; Lt = *Liriodendron tulipifera*; Md = *Malus domestica*; Ms = *Miscanthus sinensis*; Mt = *Medicago truncatula*; Nt = *Nicotiana tabacum*; Os = *Oryza sativa*; Pg = *Panax ginseng*; Ph = *Petunia hybrida*, Pt = *Pinus taeda* or *Populus trichocarpa* (marked in table); Tv = *Toxicodendron vernicifluum*; Va = *Vitis amurensis*; Vl = *Vitis labrusca*; Vr = *Vitis riparia*; Vv = *Vitis vinifera*; Zm = *Zea mays*.

A) ABC-transporters

Protein	Accession number	Publication (if not direct databank submission)
AtFRD3	NP_187461.1	Durrett et al. (2007)
AtPDR-typeABC8	NP_176196.1	Stein et al. (2006)
AtTT12	NP_191462.1	Debeaujon et al. (2001)
BrTT12	ACJ36213.1	Chai et al. (2009)
MdTT12-1	ADO22709.1	Frank et al. (2011)
MdTT12-2	ADO22711.1	Frank et al. (2011)
MtMATE1	ACX37118.1	Zhao et al. (2011)
MtMATE2	HM856605.1	Zhao et al. (2011)
NtMATE1	BAF47751.1	-
NtMATE2	BAF47752.1	-
NtMATE-A	CAQ51477.1	Morita et al. (2009)
OsTT12	ABA99853.1	-
PgPDR-typeABC3	AGT28055.1	Zhang et al. (2013)
VvABC12	XP_010654716.1	-
VvABC13	XP_002273987.1	-

Protein	Accession number	Publication (if not direct databank submission)
VvABCC1	NP_001290005.1	Francisco et al. (2013)
VvAM1	ACN91542.1	Gomez et al. (2009)
VvAM3	ACN88706.1	Gomez et al. (2009)
VvMATE1	XP_002282907.1	Pérez-Díaz et al. (2014)
VvMATE2	XP_002282932.1	Pérez-Díaz et al. (2014)
ZmMDR	AAT37905.1	Goodman et al. (2004)

B) Glycosyltransferases

Protein	Accession number	Publication (if not direct databank submission)
AtGT	AT5G65550	Brugliera et al. (1994) Kroon et al. (1994)
AtHGT	NP_188813.1	Zhao et al. (2007)
AtRT	NP_192016.1	Yonekura-Sakakibara et al. (2007)
AtUGT73B5	NP_179150.3	-
GvGT	ABK76266.1	Meesapyodsuk et al. (2007)
HsGT	XP_011512261.1	-
PgGT	AKA44597.1	Jung et al. (2014)
PhGT	CAA81057.1	Brugiera et al. (1994)
PtRT (Populus trichocarpa)	XP_002315125.2	-
VaGlu1	CZS70601.1	Kiselev et al. (2017)
VlRSGT	ABH03018.1	Hall and De Luca (2007)
VvGT1	XP_002274256.1	Khater et al. (2012)
VvGT2	XP_002285379.1	Khater et al. (2012)
VvGT3	NP_001267849.1	Khater et al. (2012)
VvGT4	XP_019072060.1	-
VvGT5	XP_019073832.1	-
VvGTF1	XP_002277035.1	Ono et al. (2010)
VvGTF6	NP_001267832.1	Ono et al. (2010)
VvGTF7	XP_002271025.1	Ono et al. (2010)
VvROMT	CAQ76879.1	Schmidlin et al. (2008)

C) Laccases

Protein	Accession number	Publication (if not direct databank submission)
VvLAC_(Pinot_noir)	XP_002264394.3	-
VvLAC14_(Chardonnay)	RVW69549.1	-
VrLAC14-like	XP_034676597.1	-
TvLAC	BAB63411.2	Nitta et al. (2002)
PtLAC1 (Pinus taeda)	AAK37823.1	Sato et al. (2001)
PtLAC2	AAK37824.1	Sato et al. (2001)
PtLAC3	AAK37825.1	Sato et al. (2001)
PtLAC4	AAK37826.1	Sato et al. (2001)
PtLAC5	AAK37827.1	Sato et al. (2001)
PtLAC6	AAK37828.1	Sato et al. (2001)
PtLAC7	AAK37829.1	Sato et al. (2001)
PtLAC8	AAK37830.1	Sato et al. (2001)
OsLAC1	AAC04576.2	-
LtLAC1	AAB17191.1	LaFayette et al. (1999)
LtLAC2	AAB17192.1	LaFayette et al. (1999)
LtLAC3	AAB17193.1	LaFayette et al. (1999)
LtLAC4	AAB17194.1	LaFayette et al. (1999)
AtLAC	AAO50504.1	-
AtTT10	NP_199621.2	Pourcel et al. (2005)
VvPPO	RVX08544.1	-
VvStOXY1	XP_002282111.2	-
VvStOXY2	XP_003634362.2	-
MsLAC1	QED40958.1	He et al. (2019)
BcLAC2	AAK77953.1	Schouten et al. (2002)
BsLAC	AFQ56549.1	-
EcMCO	WP_001189647.1	Singh et al. (2011)

5 List of abbreviations

%	percent
°C	Degrees celsius
2YT	yeast extract and tryptone medium
4CL	4-coumarate:CoA ligase
A. thaliana or At	*Arabidopsis thaliana*
A. tumefaciens	*Agrobacterium tumefaciens*
ABC	ATP binding cassette
ABTS	2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)
ACC	acetyl-CoA carboxylase
Amp	ampicillin
ANOVA	analysis of variance
ANS	anthocyanidin synthase
APS	ammonium persulfate
ATP	adenosine triphosphate
Avr	avirulence factor
Bc	*Botrytis cinerea*
bHLH	basic helix–loop–helix
BP (reaction)	Gateway reaction to create entry clone
Br	*Brassica rapa*
C4H	cinnamate 4-hydroxylase
Carb	carbenicillin
ccDB (gene)	control of cell death B (gene in the CcdA/CcdB Type II Toxin-antitoxin system in Gateway cloning)
cDNA	complementary DNA
CHI	chalcone isomerase
CHS	chalcone synthase
CoA	coenzyme A
CR	crude extract
cv	cultivar
DAD	diode array detector
DAMP	damage associated molecular pattern
DFR	dihydroflavonol reductase

DHA	3,5-dihydroxyanisole
DNA	desoxyribonucleic acid
dNTP	desoxyribonucleiotide
DTT	dithiothreitol
E. necator	*Erysiphe necator*
EGTA	ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
ETI	effector triggered immunity
F3H	flavonoid 3' hydroxylase
FLS	flavonol synthase
GC	Grape Cormier
GFP	green fluorescent protein
GLUC	betaglucanases
GT	glycosyltransferase
Gv	*Gypsophila vaccaria*
his	Histidine tags
hpi	Hours post infection
HPLC	high-performance liquid chromatography
Hs	*Homo sapiens*
IPTG	isopropyl β-D-1-thiogalactopyranoside
Kana	kanamycin
LAC	laccase
LB	Lysogeny broth
LR (reaction)	Gateway reaction to create destination vector
Lt	Liriodendron tulipifera
M	molarity
m/z	mass charge ratio
MA	microarray
MAPK	mitogen-activated protein kinase
Md	*Malus domestica*
min	minutes
mRNA	messenger RNA
Ms	*Miscanthus sinensis*
Mt	*Medicago truncatula*
MYB	myeoblastosis
NB-LRR	nucleotide binding and leucin rich repeat domains

Nt	*Nicotiana tabacum*
oligo dT	oligonucleotide of thymine repeats
ORF	open reading frame
Os	*Oryza sativa*
OXY	oxidase
P. viticola	*Plasmopara viticola*
PA	proanthocyanidins
PAA	polyacrylamide
PAL	phenylalanine ammonia lyase
PAMP	pathogen associated molecular pattern
PCD	programed cell death
PCR	polymerase chain reaction
Pg	*Panax ginseng*
Ph	*Petunia hybrida*
PR	pathogenesis-related protein
PRR	pattern recognition receptor
Pt	*Pinus taeda* or *Populus trichocarpa*
PTI	pathogen triggered immunity
qRT-PCR	quantitative real time PCR
Ren	Resistance to *Erysiphe necator*
R-genes	Resistance genes
Rif	rifampicin
RNA	ribonucleic acid
ROMT	resveratrol-O-methyltransferase
ROS	reactive oxygen species
rpm	revolutions per minute
Rpv	resistance to *Plasmopara viticola*
SDS	sodium dodecyl sulfate
sec	second
SN	supernatant
SNP	single nucleotide polymorphism
SOC	super optimal broth with added glucose
Spec	spectinomycin
STS	stilbene synthase
TAE	tris acetate EDTA buffer

TE	tris-EDTA buffer
TEMED	tetramethylethylenediamine
TF	transcription factor
Ti plasmid	tumor inducing plasmid
tt	transparent testa
Tv	*Toxicodendron vernicifluum*
UFGT	UDP-Glc:flavonoid-3-O-glucosyltransferase
UHPLC	ultra-high-performance liquid chromatography
UV	ultraviolet
V. vinifera or Vv	*Vitis vinifera*
v/v	volume per volume
Va	*Vitis amurensis*
Vl	*Vitis labrusca*
Vr	*Vitis riparia*
w/v	weight per volume
WDR	WD repeats
YEB	yeast extract beef broth
YPB	yeast extract peptone dextrose
Zm	*Zea mays*

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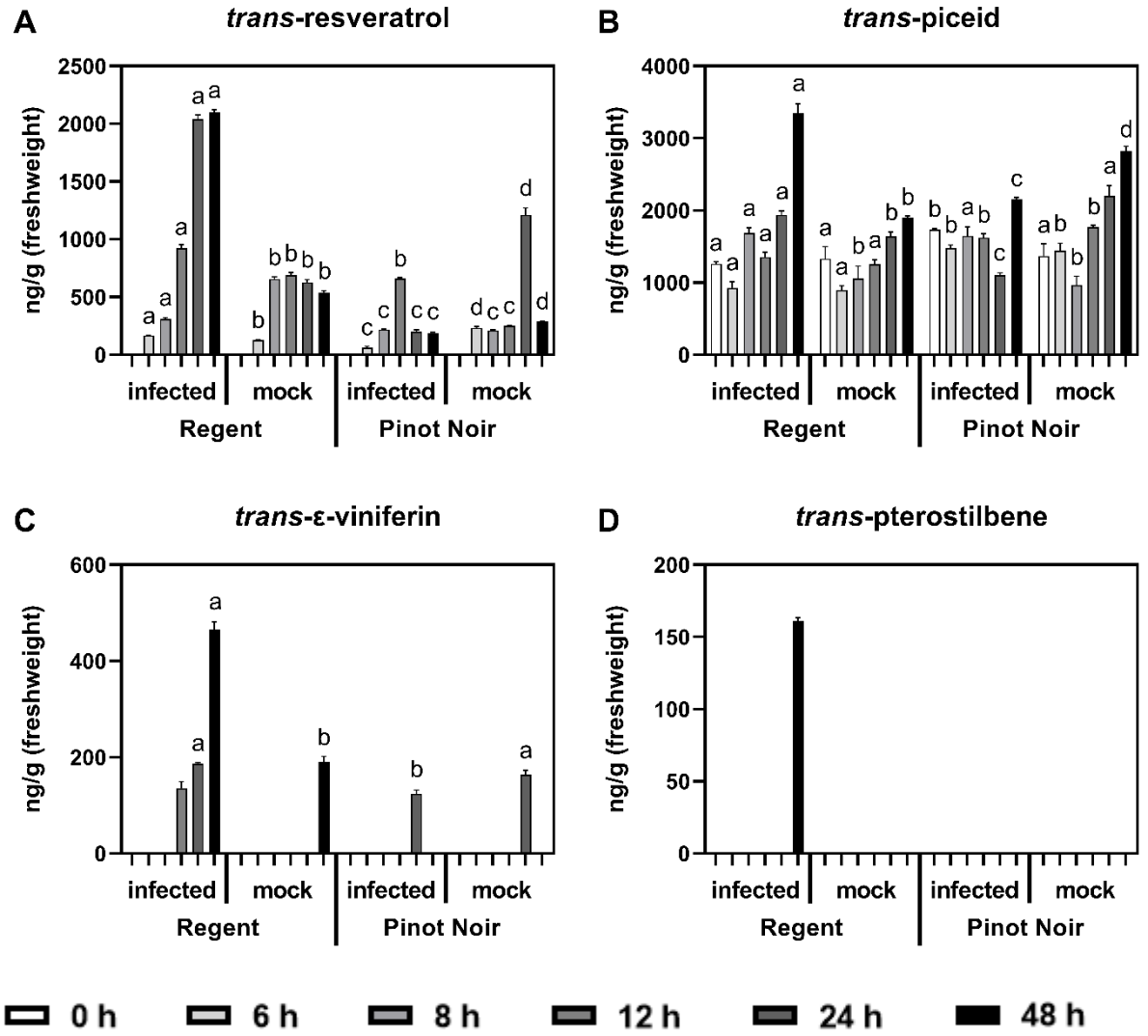
7 Appendix

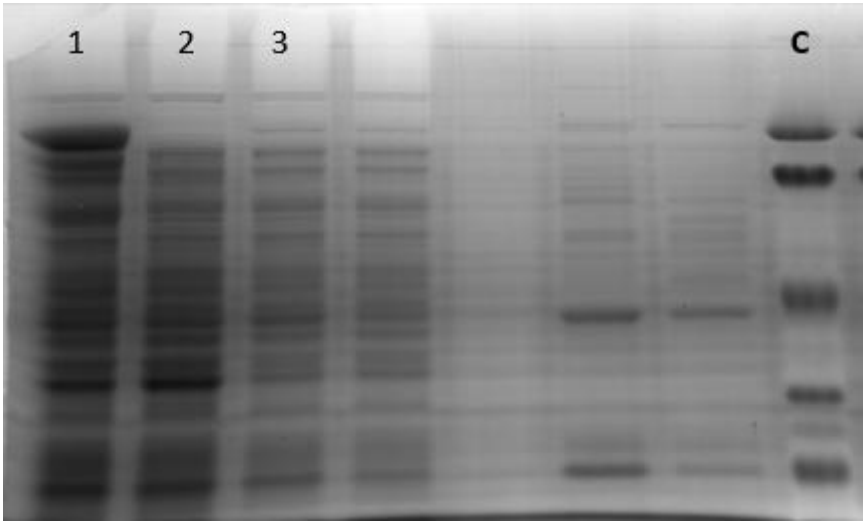


Supplemental Figure 1: Amount of selected stilbenes in *V. vinifera* leaf discs of the 'Regent' and 'Pinot Noir' varieties during *P. viticola* infection.

The amount of *trans*-resveratrol (A), *trans*-piceid (B), *trans*-ε-viniferin (C) and *trans*-pterostilbene (D) in *V. vinifera* cv. 'Regent' and 'Pinot Noir' leaf discs infected with *Plasmopara viticola* or mock treated was determined by HPLC. The leaf discs were sampled 0 h, 6 h, 8 h, 12 h, 24 h and 48 h after infection or water treatment (mock) (see figure legend). The graphs show the amount of stilbenes in ng/g (freshweight), expressed as mean values of three replicates (n=3) and error bars indicating standard deviation. An analysis of variance (ANOVA) was conducted to determine the effects of cultivar and treatment on the content of *trans*-resveratrol, *trans*-piceid, *trans*-ε-viniferin or *trans*-pterostilbene content of the grape berries. The values were compared by Tukey's HSD test, different letters (a-d) indicate significant differences between cultivar and treatment within sampling time (hpi) column ($p < 0.05$). For statistical comparison between the sampling time (hpi) within one cultivar and treatment group, see Figure 11.

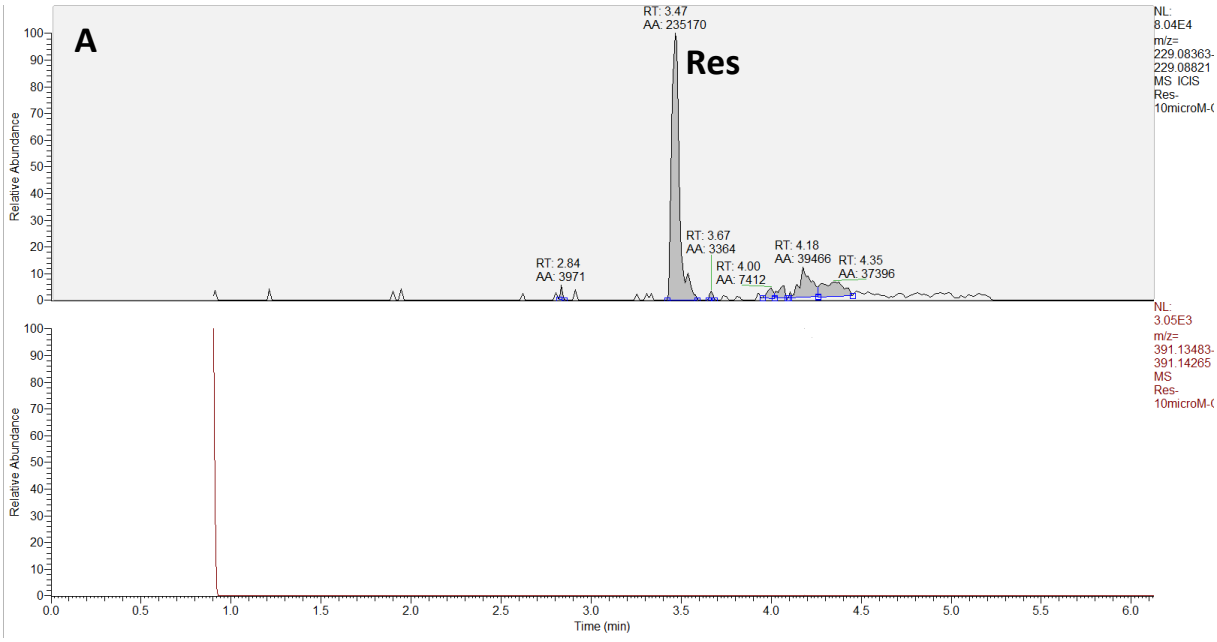
Supplemental Figure 2: Culture growth, measured as OD_{600}, of the candidate gene containing *S. cerevisiae* cultures after induction.

S. cerevisiae strains expressing grapevine genes for resveratrol synthesis and transport, provided by the cooperation partner Evolva were transformed with the candidate genes *VvGT2*, *VvGT4*, *VvGT5* and *VvLAC*. The cells were grown in induction medium or non-inducing control medium, optical density was measured at 600 nm wavelength at the 0 h, 2 h, 6 h and 24 h after induction-timepoints before the stilbenes were extracted and measured by HPLC.



Supplemental Figure 3: Uncut PAA-gel of protein extract of *E.coli* strain expressing *VvGT5* (see Figure 15).

Uncut version of Figure 15 C. Lane one shows the crude extract of the induced culture (1 mM IPTG, 4 h), lane two the same culture uninduced and lane three the protein content in the supernatant of the induced culture (lane one). The rightmost lane is a marker lane, molecular weight in kDa (Pierce™ prestained protein molecular weight marker). The unmarked lanes are samples that are not described in this work.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AA: 39466
RT: 4.35
AA: 37396
NL:
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m/z=
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229.08821
MS ICIS
Res-
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MS
Res-
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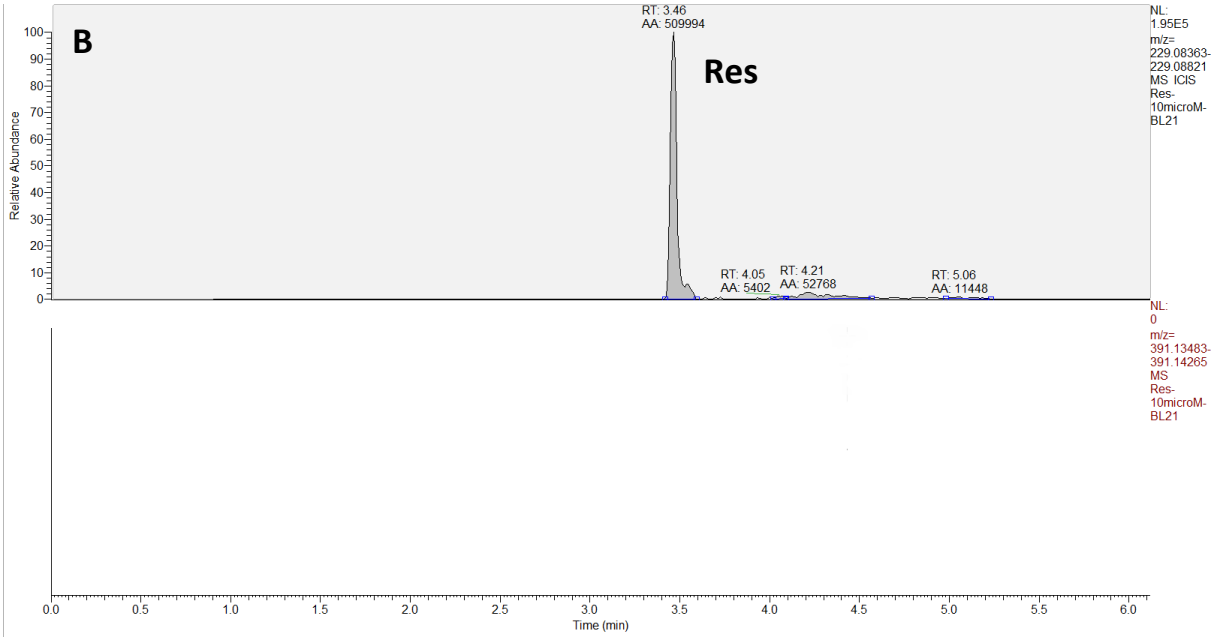
Relative Abundance
Time (min)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Res-
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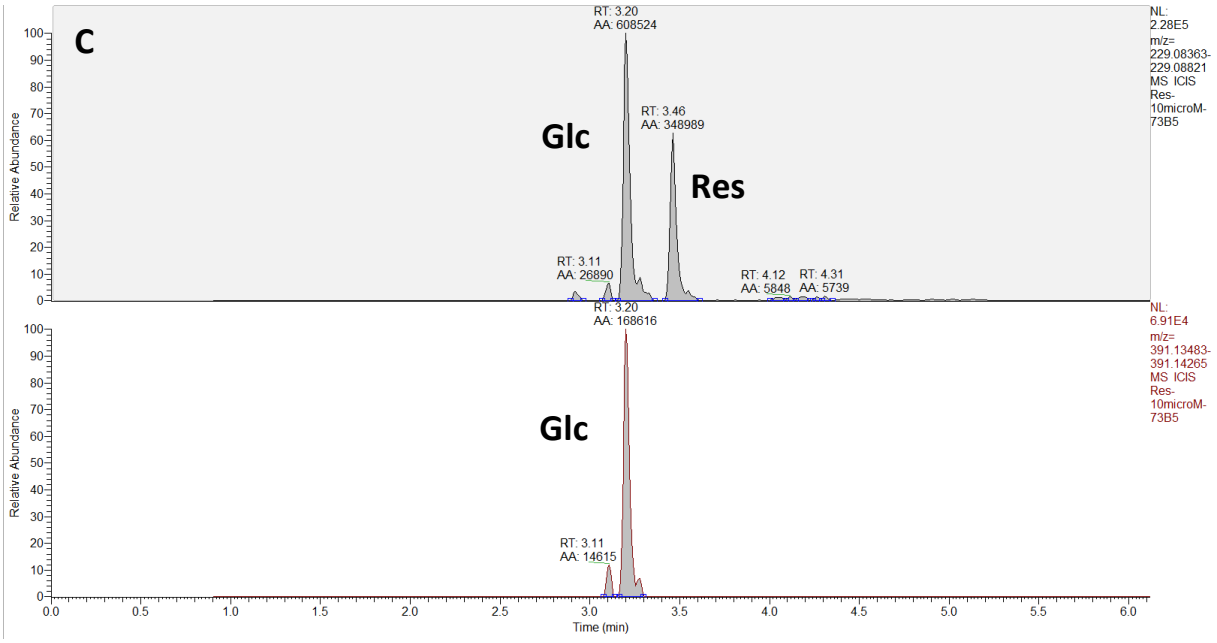
BL21
Relative Abundance
Time (min)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Glc
Res
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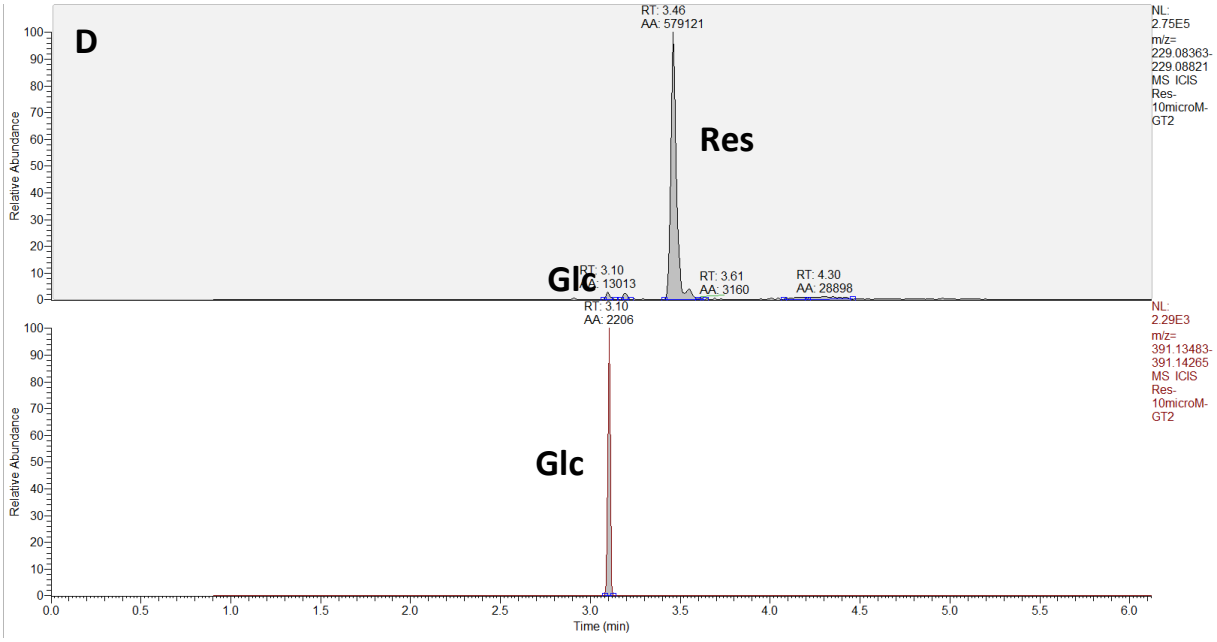
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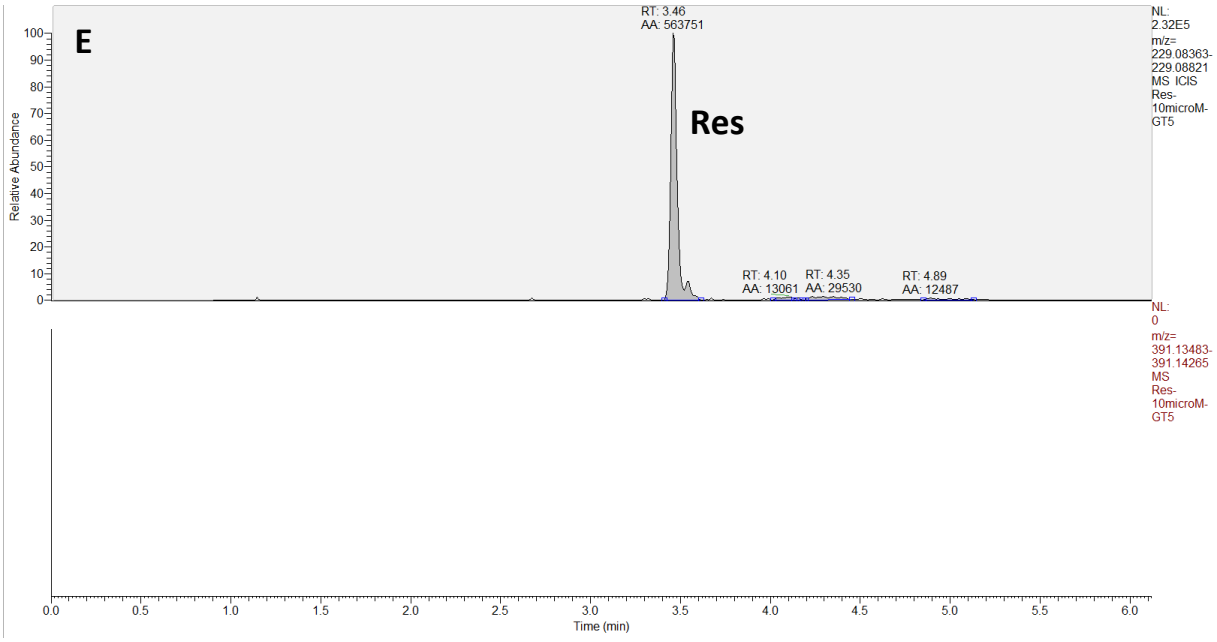
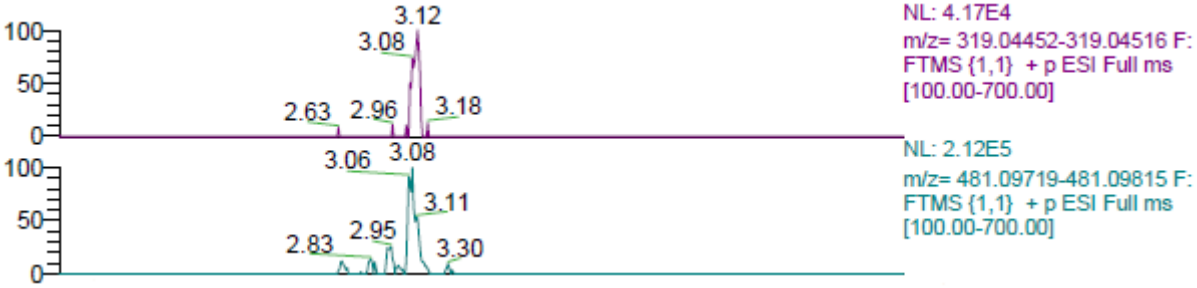
Time (min)



Supplemental Figure 4: Relative amount of selected *trans*-resveratrol measured by UHPLC-MS after protein extraction and enzymatic assay.

The glycosyltransferases *At73B5*, *VvGT2* and *VvGT5* were transformed into BL21 DE3 *E.coli* strains. *Trans*-resveratrol (10 µM) as well as UDP-glucose (1 mM) was provided to the transformed strains for an overnight enzymatic assay. The amount of remaining substrate was measured by UHPLC-MS and is displayed as raw-data peaks. The panels show the signal for the mass of *trans*-resveratrol in the respective upper panel (grey background) and for glycosylated *trans*-resveratrol in the respective lower panel (white background). The enzymes are: control (none; A), BL21 cell extract (B), the same extract with At73B5 expression (C), VvGT2 expression (D) and VvGT5 expression (E). The peaks of the two different masses cannot directly be compare due to the different "response factor" of the molecules in the mass detector. Furthermore, a peak can be seen at the retention time of the glycosylated *trans*-resveratrol but in the *trans*-resveratrol mass spectrum, which indicated that during ionization, some glucose molecules are cleaved of and the "free" *trans*-resveratrol is measured here. For further analysis, it was investigated, whether one of the glycosylated peaks was present (yes/no answer) and the amount of glycosylation could then be estimated by the decrease of the original *trans*-resveratrol peak. The graphs depict one experiment, which are representative of 2-3 independent experiments with a fresh batch of the same stain, which resulted in similar trends but varying raw signal.

A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[100.00-700.00]
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3.08
3.11
2.83
2.95
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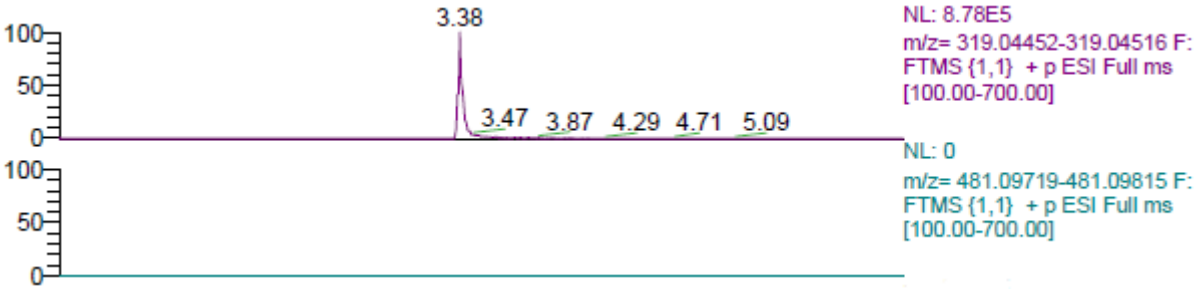
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5.09
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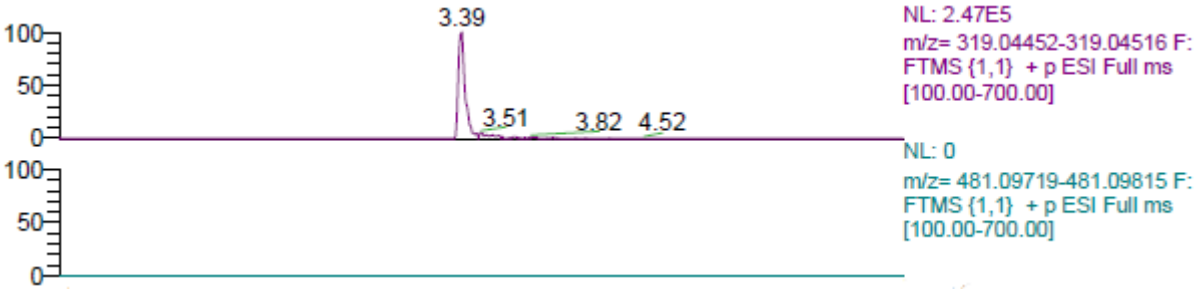
C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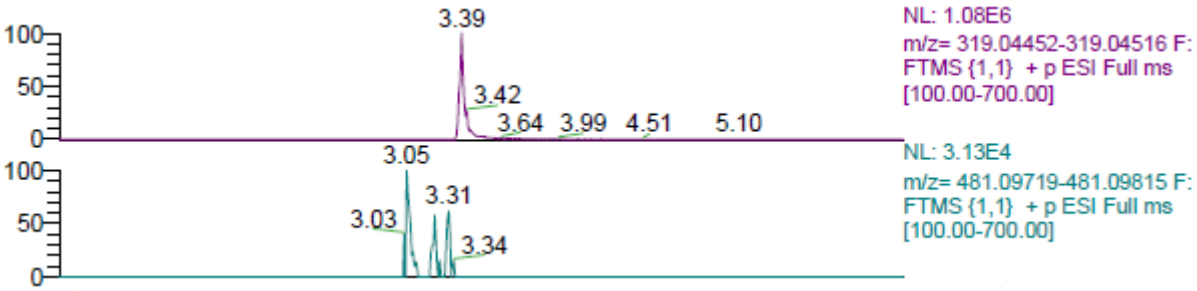
D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3.31
3.03
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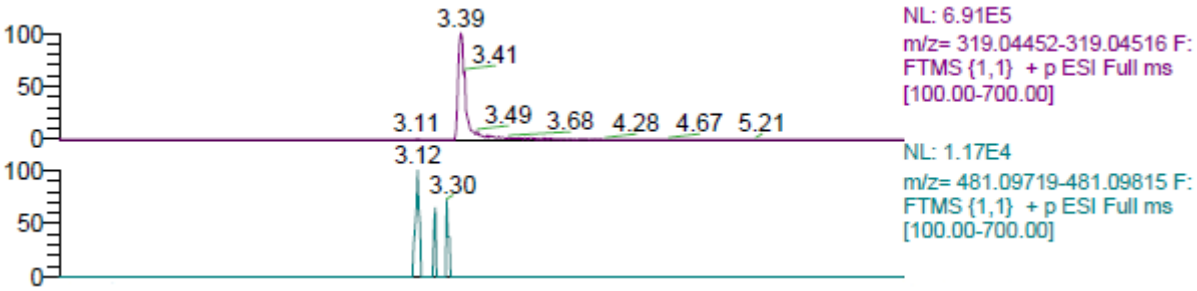
E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3.30
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FTMS {1,1} + p ESI Full ms
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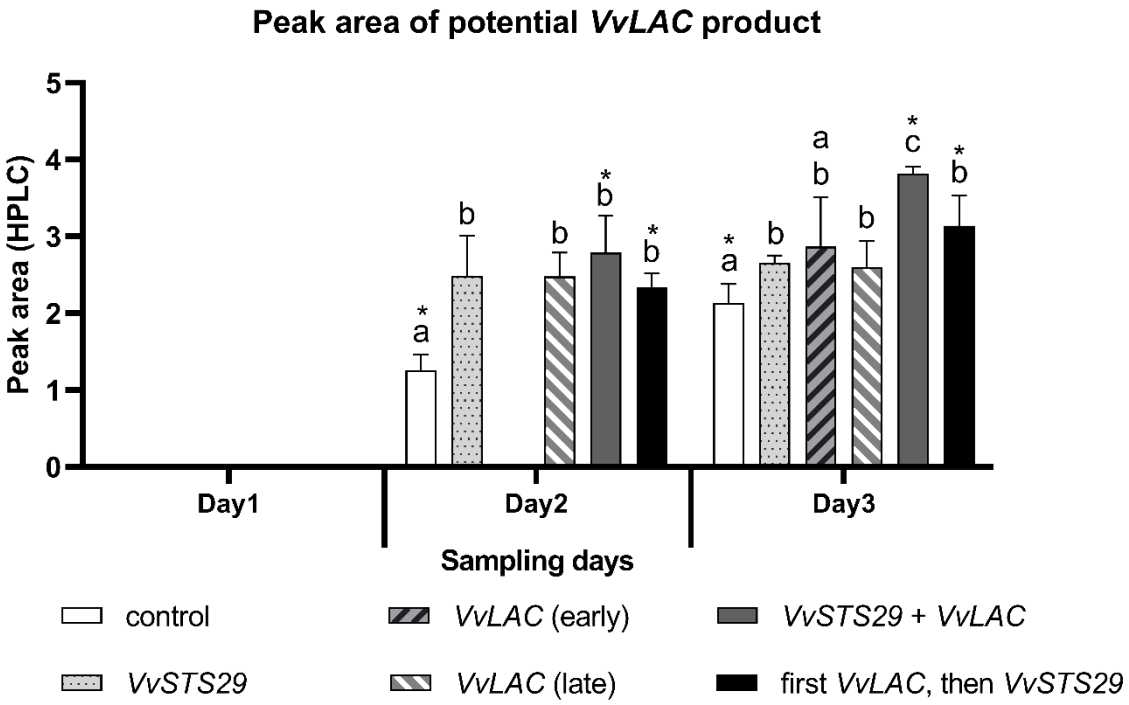
Supplemental Figure 5: Additional potential substrates for the investigated glycosyltransferases as discovered in an enzymatic assay with a substrate mix, measured by UHPLC-MS.

The glycosyltransferases At73B5, VvGT2 and VvGT5 transformed into a BL21 DE3 *E.coli* strain, which then were provided with a substrate mix containing 17 polyphenols and 11 further common substrates in a 10 µM concentration as well as UDP-glucose (1 mM). Besides the previously investigated substrates (Figure 16), caffeic acid, ethyl-gallate, naringenin and myricetin were also glucosylated (see Table 4).

The graphs A-E depict the chromatograms for myricetin (violet) and its glucoside (blue) in comparison between the used enzymes or controls as an example of the substrates: At73B5 (A), BL21 empty cells (B), control without cells (C), VvGT2 (D) and VvGT5 (E).



Supplemental Figure 6: Peak areas of a potential product of VvLAC in *N. benthamiana*.

The amount of an unidentified substance in *N. benthamiana* after infiltration with *VvSTS29* and *VvLAC,* measured by HPLC. The four week old plants were infiltrated with pEarleyGate203:VvSTS29 and pB7WG2D:VvLAC in *A. tumefaciens* (C58C1) and grown for 48 h in the greenhouse "Early *VvLAC*" infiltration occurred one day before the regular infiltrations. Samples were taken 48 h (Day 1), 72 h (Day 2) and 96 h (Day 3) after infiltration. Since the substance was not identified, no quantification could be performed. The bars express a mean value of three replicates (n=3) and error bars indicate standard deviation. An analysis of variance (ANOVA) was conducted to determine the effects of sampling time (days) and transformed gene on the content of the unknown substance similar to *trans*-ε-viniferin of the medium. The values were compared by Tukey's HSD test, different letters (a-c) indicate significant differences between transformed gene line within one sampling timepoint and asterisks indicate significant difference between the same sampling timepoint within one gene-line ($p < 0.05$).

8 Acknowledgements

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