New Genes from an Ancient Plant

– Mining *Vitis sylvestris*

Zur Erlangung des akademischen Grades eines

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Hiermit erkläre ich, dass ich die vorliegende Dissertation, abgesehen von der Benutzung der angegebenen Hilfsmittel, selbständig verfasst habe.

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Karlsruhe, im Juni 2015

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Abbreviations

Avr gene/proteins, avirulence gene/proteins

BAK1, BRI1 associated receptor kinase 1

CC, coiled-coil

CTAB, cetyltrimethylammonium bromide

CWRs, crop wild relatives

DIC, differential interference contrast

DMSO, dimethylsulfoxide

DPI, diphenylene-iodonium chloride

EDS1, enhanced disease susceptibility 1

EF-Tu, elongation factor Tu

EFR, elongation factor Tu receptor

Et, ethylene

ETI, effector-triggered immunity

EtOH, ethanol

FLS1, flavonol synthase 1

FLS2, flagellin-sensitive 2

GC, Grape Cormier medium

$GdCl_3$, gadolinium chloride

HPLC, high performance liquid chromatograph

HR, hypersensitive response

JA, jasmonic acid

LC-MS, liquid chromatography-mass spectrometry

LRR, a leucine-rich repeat region

MAMPs, microbe-associated molecular patterns

MAPK, mitogen-activated protein kinase

MeJA, methyljasmonate

NB-LRR, nucleotide binding-leucine rich repeat

NDR1, non-race specific disease resistance 1

PAMPs, pathogen associated molecular patterns

PCA, principal component analysis

PCD, programmed cell death

PD98059, 2-(2-Amino-3-methoxyphenyl)-4H-1-benzopyran-4-one

PKSs, type III polyketide synthases

PR genes/proteins, pathogenesis-related genes/proteins

PRRs, pattern recognition receptors

PTI, PAMP-triggered immunity

R genes/proteins, resistance genes/proteins

RLK, a receptor-like kinase domain

ROS, reactive oxygen species

ROMT, resveratrol *o*-methyltransferase

Rpv3, resistance to *P. viticola* 3

RT-PCR, semi-quantitative reverse transcription-PCR

SA, salicylic acid

SAR, systemic acquired resistance

SSR, simple sequence repeat

STAND, signal transduction ATPases with numerous domains

T3SS, type III secretion systems

TFs, transcription factors

TIR, Toll/IL-1 receptor

Tween® 20, polyoxyethylene-20-sorbitan monolaurate

***V. sylvestris*,** European Wild Grape *V. vinifera* L. *ssp. sylvestris* Hegi

Zusammenfassung

Stilbene als bedeutende Sekundärmetabolite sind die zentralen Phytoalexine der Weinrebe und daher ein wichtiges Element der basalen Immunität. In dieser Arbeit untersuchten wir mögliche genetische Variabilität hinsichtlich der Stilbenbildung bei *Vitis sylvestris*, dem Vorfahr der Kulturrebe als mögliche Quelle für die Resistenzzüchtung. Wir finden bei *V. sylvestris* beträchtliche Unterschiede in Bezug auf die Induzierbarkeit von Stilbenen und zeigen genotypische Unterschiede in Menge und Zusammensetzung von Stilbenen, die in Antwort auf einen UV-C Puls gebildet werden. Dabei entdeckten wir zwei Stilben "chemovare": eine Gruppe von Wildreben bildet Stilbene schnell und stark, fast ausschließlich in From von nicht-glycosyliertem Resveratrol und Viniferin. Die zweite Gruppe akkumuliert weniger Stilbene und diese vorwiegend als Piceatannol and glycosyliertes Piceid. Für alle 86 Genotypen beobachteten wir ein zeitabhängiges Muster der Stilbenbildung: während Piceid, Resveratrol und Piceatannol früh nachweisbar waren, fanden sich die Viniferine erst später. Wir fanden weiter, dass die genotypischen Unterschied in der Akkumulation von Stilbenen mit vorausgehenden entsprechenden Unterschieden in der Akkumulation von Transkripten korrelierten, die mit Stilbensynthese zusammenhängen: Phenylalanin-Ammonium-Lyase (*PAL*), Stilben-Synthase (*StSy*), Resveratrol-Synthase (*RS*) und Chalcon-Synthase (*CHS*). Dieselbe Sammlung von Wildreben wurde hinsichtlich ihrer Empfindlichkeit gegenüber dem Falschen Mehltau der Weinrebe (*Plasmopara viticola*) durchmustert und zeigte hier ebenfalls eine große Bandbreite. Die Subpopulation von Genotypen mit starker Stilben-Induzierbarkeit war signifikant weniger anfällig als die stilben-armen Genotypen. An entsprechend ausgewählten Genotypen konnten wir zeigen, dass die Induzierbarkeit des Schlüsselenzyms Stilben-Synthase durch UV mit der Induzierbarkeit durch das Pathogen korrelierte.

Im nächsten Schritt fragten wir, ob die starke Induktion von Stilben-Synthase Transkripten bei Hoe29 (einem repräsentativen Genotypen der stilbenreichen Gruppe) mit einer erhöhten Induktion der Transkriptionsfaktoren *MYB14* und *MYB15* zusammenhängt. Über *next-generation sequencing* wurden spezifische Unterschiede im *MYB14* Promotor

von Hoe29 gefunden, die durch direkte Klonierung bestätigt wurden. Im Gegensatz dazu zeigten sich bei *MYB15* keine offensichtlichen Unterschiede. Die stilbenreichen *sylvestris* Genotypen Hoe29 and Ke83 wiesen gewisse Übereinstimmungen auf (differierten jedoch untereinander in anderen Domänen), die im Promotor der Kultursorte Augster Weiss (die nur wenig Stilbene akkumuliert) fehlen. Wir können zeigen, dass für diese Genotypen die Unterschiede hinsichtlich der Induzierbarkeit der Stilbene Synthase mit Unterschieden hinsichtlich der Induzierbarkeit von *MYB14* Transkripten korrelieren. Um die funktionelle Bedeutung dieser spezifischen Promotor-Motive besser zu verstehen und die stromauf gelegenen Signale zu kartieren, setzten wire in Promotor-Reporter-Testsystem ein und konnten zeigen, dass beide *sylvestris* Promotoren (aber nicht der promotor des schwachen Stilbenbildners Augster Weiss) auch in Zellkultur durch UV Licht induziert werden. Allerdings konnte nur der *MYB14* promotor aus Hoe29 durch flg22 und Jasmonsäure induziert werden, was darauf hindeutet, dass das Allel von Hoe29 durch den PTI Signalweg angesteuert wird. Im Gegensatz dazu, war der *MYB14* Promotor aus Ke83 zwar durch UV Licht induzierbar, nicht aber durch Aktivierung der PTI. Dieser Befund ist konsistent mit unserer Beobachtung, dass bei beiden Genotypen die *StSy* Transkripte zwar durch UV Licht stark induziert wurden, während bei Infektion mit *P. viticola* nur bei Hoe29, nicht aber bei Ke83 eine solche Induktion zu beobachten war. Unsere Daten legen nahe, dass eine spezifische Region im Promotor eines spezifischen *sylvestris* Allels von *MYB14* Potential als Kandidat für die Resistenzzüchtung besitzt.

Abstract

Stilbenes, as important secondary metabolites of grapevine, represent central phytoalexins and therefore constitute an important element of basal immunity. In this study, we probed for potential genetic variation in *Vitis sylvestris*, the ancestor of cultivated grapevine with respect to their output of stilbenes and potential use for resistance breeding. We have identified considerable variation in stilbene inducibility in *V. sylvestris*. We show genotypic differences in abundance and profiles of stilbenes that are induced in response to a UV-C pulse. Two clusters of stilbene "chemovars" emerged: one cluster with quick and strong accumulation of stilbenes, almost exclusively in form of the non-glycosylated forms resveratrol and viniferin. The second cluster accumulated less stilbenes and relatively high proportions of piceatannol and the glycosylated piceid. For all 86 genotypes, we observed a time-dependence of the stilbene pattern: piceid, resveratrol and piceatannol accumulated earlier, whereas the viniferins were found later. We further observed that the genotypic differences in stilbene accumulation were correlated by a preceding differential accumulation of the transcripts for stilbenes-related genes: phenylalanine ammonium lyase (*PAL*), stilbene synthase (*StSy*), resveratrol synthase (*RS*), and chalcone synthase (*CHS*). A screen of the population with respect to susceptibility to Downy Mildew of Grapevine (*Plasmopara viticola*) revealed considerable variability. The subpopulation of genotypes with high stilbene inducibility was significantly less susceptible as compared to low-stilbene genotypes, and for representative genotypes we could show that the inducibility of stilbene synthase to UV correlated with the inducibility by the pathogen.

In the next step we asked, whether the strong induction of stilbene synthase transcripts in Hoe29 (a representative genotype of the strong stilbene producers) might be linked with an elevated induction of the transcription factors *MYB14* and *MYB15*. Specific differences were discovered by next-generation sequencing and confirmed by cloning for the *MYB14* promotor of Hoe29, whereas the *MYB15* did not reveal obvious changes. The strong stilbene producers Hoe29 and Ke83 (both *sylvestris* genotypes) harbour certain common domains (whereas they differ in others), which were absent from the promotor of the

cultivated variety Augster Weiss (that is a weak stilbene producer). We can show that the differences in stilbene synthase inducibility between these genotypes correlate with differences in the induction of *MYB14* transcripts. To understand the functional relevance of these specific promotors and to map the upstream signalling, we employed a promotor-reporter assay and showed that both *sylvestris* promotors (but not the promotor from the weak stilbene producer Augster Weiss) are induced by UV light in cell culture, however, only the *MYB14* promotor from Hoe29, was induced by flg22 and jasmonic acid indicating that the allele of Hoe29 was under control of PTI signalling. In contrast, the *MYB14* promotor from Ke83, although inducible by UV light, was not induced in the context of PTI. This is consistent with our findings, where *StSy* transcripts were strongly induced by UV light in both genotypes, whereas for infection by *P. viticola* induction was observed only in Hoe29, but not in Ke83. Our data suggest that a particular region in the promotor of a specific *sylvestris* allele of *MYB14* harbours potential as candidate target for resistance breeding.

1. Introduction

1.1 Multi-level defence mechanism in plants

In their natural environment, plants are exposed to numerous pathogens, including viruses, bacteria, fungi, protozoa, plants and animals (Glazebrook, 2005). During the longlasting evolutionary interaction with these pathogens, plants have formed multi-level, effective defence mechanism against the pathogen infection. The first defence line of plants is physical barriers. For example, the cell wall, wax layers, and cuticle can prevent the pathogens from entering cells (Göhre and Robatzek, 2008). These physical barriers already existed very early in the evolution history of plants and thus represent a constitutive first line of defence (Göhre and Robatzek, 2008), which provides a basic and broad-range of resistance.

The second line of defence is carried by the innate immune system of plants (Zipfel, 2008; Vance *et al*., 2009; Zhou and Chai, 2008; Zhang *et al*., 2008). When pathogens manage to overcome these physical barriers, chemical barriers will be installed. Enzymatic and antibacterial compounds secreted by the plant, such as phenols, saponins, unsaturated lactone, or organosulfur compounds provide efficient chemical defence against pathogen invasion (Zipfel, 2008). Recognition of pathogens by receptors in the plasma membrane will activate signalling and induction of defence genes, some of which encode the ability to synthetise these compounds. As explained in the next chapter, some pathogens can successfully break through this second line of defence and enter the cytoplasm to suppress chemical plant defence responses and reprogramme their host to provide the nutrients for their own survival. In some host plants that have undergone a coevolutionary history with the pathogen, the pathogen will now face the second line of innate immunity (Göhre and Robatzek, 2008; Zhou and Chai, 2008; Liu *et al*., 2011). These adapted plants can identify the invasion of (non-self) pathogens through specific, cytoplasmic receptors, and the recognition of the pathogen signal will reinstall defence-related signal transduction, expression of defence genes, and often programmed cell death as efficient tool to kill the invader (Jones and Takemoto, 2004).

1.2 Plant innate immune system

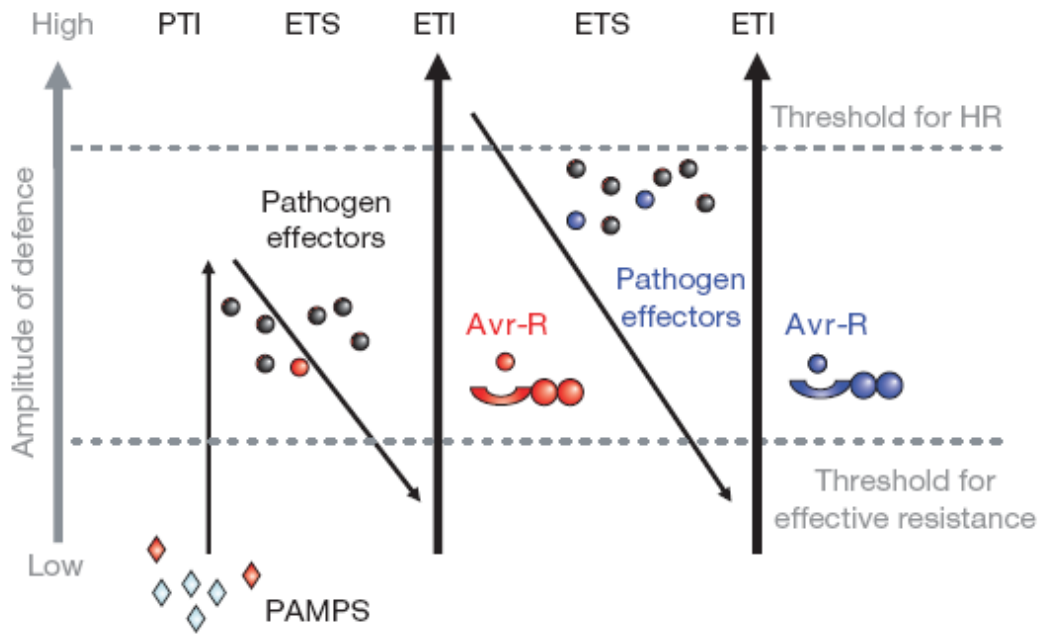


Fig.1 The "zigzag model" illustrates the quantitative output of plant immune system (Jones and Dangl, 2006). PAMPs, pathogenesis-associated molecular patterns; PTI, PAMP-triggered immunity; ETS, effector-triggered susceptibility; ETI, effector-triggered immunity; Avr, avirulences; R, resistance proteins; HR, hypersensitive response.

The plant innate immune system has two distinct layers to encounter pathogen invasion (Jones and Dangl, 2006). The first layer is based on the perception of pathogen-associated molecular patterns (PAMPs) at the plasma membrane (Nürnberger *et al*., 2004; Bittel and Robatzek, 2007; Naito *et al*., 2008) through pattern-recognition receptors (PRRs). At this stage, plants recognize and respond to ubiquitous molecules in pathogenic microorganisms in a so called PAMP-triggered immunity, PTI (Jones and Dangl, 2006; Boller and He, 2009; Ausubel, 2005). The second layer starts intracellularly and mainly relies on resistance (*R*) proteins. These proteins are mostly nucleotide binding-leucine rich repeat (NB-LRR). The successfully invading pathogens inhibit the receptor activity on the cell membrane through secreting toxic effectors using type III secretion systems (T3SS) and diverting host nutrients for their own survival within the interior of the plant cell (Abramovitch *et al*., 2006; Block *et al*., 2010; Kay and Bonas, 2009). Different

NB-LRR can directly or indirectly recognize different effectors and initiate defence responses named effector-triggered immunity, ETI (Takken and Tameling, 2009; Chung *et al*., 2011; Padmanabhan *et al*., 2009). The molecular mechanisms of the two layers are not identical but partially overlap. Thus, the interaction between plants and pathogenic microorganisms is complex. ETI is an accelerated and amplified PTI response (Chisholm *et al*., 2006) and usually accompanied by a hypersensitive response (HR) at the infection site. Natural selection drives pathogens to avoid ETI either by shedding or diversifying the recognized effectors, or by acquiring additional effectors to suppress ETI. On the side of the host, natural selection results in new *R* specificities so that ETI can be reinstalled. This process of the interaction and coevolution between plants and pathogens is visualised by the "zigzag model" in Fig. 1 (Jones and Dangl, 2006).

1.2.1 PAMPs and recognition

PAMPs are conserved molecules existing in the surface of pathogenic microorganisms. Because these molecules are widely abundant in microbes (Zipfel, 2008), they are also referred to as microbe-associated molecular patterns (MAMPs). Classical examples include the elongation factor Tu (EF-Tu), bacterial flagellin, lipopolysaccharide, peptidoglycans, xylanase, and β-glucans (Naito *et al*., 2008; van de Veerdonk *et al*., 2008). Plants perceive PAMPs through pattern recognition receptors (PRRs) on the surface of their cellular membrane. The PAMP bacterial flagellin and its corresponding receptor, the PRR flagellin sensitive 2 (FLS2) first identified in *Arabidopsis* represent the most extensively studied case. Another PAMP recognized by *Arabidopsis* is EF-Tu, which is abundant in bacteria. EF-Tu harbours highly conserved elf18 peptides, which contain 18 amino acids and are acetylated in the N-terminus. The leucine-rich repeats receptor-like kinase (LRR-RLK) EF-Tu receptor is responsible for identifying EF-Tu in *Arabidopsis* and also belongs to the same LRR subfamily as FLS2 (Kunze *et al*., 2004). The brassinosteroid-insensitive1 (BRI1)-associated receptor kinase 1 (BAK1), an universal adaptor protein in a variety of signal transduction pathways, acts also as coreceptor in PTI in a complex with FLS2 and the elongation factor receptor (EFR) (Hecht *et al*., 2001; Wang *et al*., 2008).

1.2.2 Flagellin-a typical PAMP

The flagellum is a lash-like appendage found in most bacteria. The primary role of the flagellum is locomotion (Moens and Vanderleyden, 1996). The flagellum is an important virulence factor for bacteria pathogenic to animals and plants (Finlay and Falkow, 1997), and consists of the protein flagellin. The most highly conserved peptide domain of flagellin is named flg22 and located close to N terminus of flagellin. It has been shown to be an effective elicitor to induce the defence response of tomato or other plant cells (Felix *et al*. 1999).

So far, the bacterial flagellin is the best studied example of a PAMP. FLS2 as an LRR receptor-like kinase has been found to act as receptor for bacterial flagellin in *Arabidopsis* (Gómez-Gómez and Boller, 2000). The LRR-RLK class of proteins often consists of a domain containing an extracellular leucine-rich repeat (LRR) region, a transmembrane domain, and a cytoplasmic receptor-like kinase (RLK) domain (Gómez-Gómez and Boller, 2000; Shiu and Bleecker, 2001; Fritz-Laylin *et al*., 2005). In Arabidopsis, the receptor FLS2 binds the conserved flg22 motif in the N-terminus of flagellin (Chinchilla *et al*., 2006; Felix *et al*., 1999), and similar mechanisms have also been found in tomato, tobacco and rice (Robatzek *et al*., 2007; Hann and Rathjen, 2007; Takai *et al*., 2008). This means plants perceive flagellin through FLS2 homologues, which represent an evolutionary ancient and conserved aspect of plant immunity.

Some studies have already shown that flg22 can induce the defence responses in plants (Gómez-Gómez *et al*., 1999; Zipfel *et al*., 2004; Gómez-Gómez and Boller, 2002; Nicaise *et al*., 2009) and that the peptide is more active than full-length flagellin (Felix *et al*., 1999) with respect to several responses, such as the production of ethylene, inhibition of growth, changes of ion fluxes, the production of reactive oxygen species (ROS), and the expression of some defence-related genes (Felix *et al*., 1999; Gómez-Gómez *et al*., 1999; Zipfel *et al*., 2004; Asai *et al*., 2002; Nguyen *et al*., 2010). However, there is a debate whether flg22 can induce the HR (Che *et al*., 2000; Naito *et al*., 2008).

As PAMP-induced defence response mechanisms and signal transduction pathways in plant cells provide the basis of plant defence, they have always been the key of plant

molecular pathology. Flg22 as one of the most in-depth studied PAMPs provides a valuable tool for research on plant defence. Especially the identification of the receptor FLS2 provided insight on how defence signaling interacts with other signaling events occulting in plant cells.

1.2.3 The recognition of effectors and effector-triggered immunity

Pathogenic microorganisms overcome the first line of defence in plant immune system using the effectors, and under the pressure of this selective pressure, plants have evolved receptors to identify these effectors and activate a second layer of immunity–called effector-triggered immunity (ETI). The coding products of R genes, the R proteins, are usually located in the cytoplasm, which coincides with the localization of the corresponding effectors that are encoded by so called avirulence (Avr) genes. ETI is based on the direct or indirect perception of R proteins by Avr proteins, and therefore has been traditionally described by the gene for gene concept. Most of the R proteins are nucleotide binding-leucine rich repeat (NB-LRR) proteins, which form a subgroup called STAND (signal transduction ATPases with numerous domains) family. In animals, STAND family members include immune, inflammation, and apoptosis regulators (Lukasik and Takken, 2009). The NB-LRR proteins of plants are mainly divided into two categories according to their N-terminal domain: one is the group of TIR (Toll/IL-1 receptor)-NB-LRR and another is the group of CC (coiled-coil)-NB-LRR (Dangl and Jones, 2001). The different N termini target to different downstream signal transduction components: TIR-NB-LRRs addresses enhanced disease susceptibility 1 (EDS1) and most of CC-NB-LRRs interact with the non-race specific disease resistance 1 (NDR1) proteins to activate the immune responses (Elmore *et al*., 2011). In plants, NB-LRRs can perceive the effectors and activate ETI either directly (Deslandes *et al*., 2003, Dodds *et al*., 2006; Catanzariti *et al*., 2010; Jia *et al*., 2000; Krasileva *et al*., 2010), but also indirectly (Axtell and Staskawicz, 2003; Wilton *et al*., 2010; Mackey *et al*., 2002; Shao *et al*., 2003; Abramovitch and Martin, 2005; Mucyn *et al*., 2006).

Whereas PTI is general, evolutionary ancient and active against numerous pathogens, ETI is more targeted. Under natural conditions, PTI and ETI are both involved in plant

resistance responses, and may act at the same time during the interaction between pathogens and plants. Thus, the connection between PTI and ETI are subject to our further explorations.

1.3 Signal pathways in plant basal immunity

Plants are exposed to a large range of biotic and abiotic stresses involving a series of signal transduction pathways that mediate the stress responses that have to be specific for different types of stresses.

1.3.1 Ca^{2+}- a second messenger operating in stress signalling

As early cellular response rapid changes in ion fluxes across the plasma membrane occur. These rapid responses involve an increased influx of Ca^{2+} and H^{+}, and an efflux of K^{+} (Nürnberger, 1999). Especially Ca^{2+} as secondary messenger plays an important role in plant signal transduction triggered by environmental stimulation (Harper and Harmon, 2005). The influx of Ca^{2+} is one of the earliest signal responses to pathogen attack (Ma *et al*., 2009). Under adverse conditions, the elevation of cytosolic Ca^{2+} transfers the signal to regulate the physiological reactions in plant growth, development and adaptation to stress (Bush, 1995).

Studies have shown that the influx of Ca^{2+} can induce the generation of ROS (Grant *et al*., 2000), at the same time, the generation of ROS also can induce an influx of Ca^{2+} (Torres *et al*. 2006). Ca^{2+} also can directly regulate gene expression in plants (Kim *et al*., 2009), such as the induction of pathogenesis-related (PR) genes (Lee and McNellis, 2009).

The antibiotic ionophore A23187 forms neutral complexes in a ratio of 2:1 with divalent cations. Because of its high selectivity for Ca^{2+}, it is used as an important tool to study Ca^{2+} transport across the membrane (Pfeiffer *et al*., 1974). The neutral complex of A23187 with Ca^{2+} can permeate through the cell membrane and release Ca^{2+} in the cytoplasm. This allows to trigger calcium in the absence of an external stimulus, which provides an important tool for functional analysis. Conversely, the activity of calcium influx channels can be specifically blocked by lanthanium ions, such as Gd^{3+} (Ismail *et al.*, 2014).

1.3.2 The production of reactive oxygen species (ROS)

Under physiological conditions, the generation and removal of reactive oxygen species in plant cells is balanced. But when plants suffer from biotic and abiotic stresses, such as pathogen attack (Apel and Hirt, 2004), salt stress (Ismail *et al*., 2012), and wounding, the production and metabolism of reactive oxygen species will be out of balance and oxidative stress is produced (Neill *et al*., 2002a). When oxidative stress occurs, the plant cells will produce excess ROS, such as O_2^-, H_2O_2, HO· and NO·. However, ROS are not only related with stress damage (Buchanan *et al*., 2000), but also can be used as signalling molecules causing molecular, biochemical and physiological responses in plant cells (Fath *et al*., 2002; Vranová *et al*., 2002). ROS have been proposed to be involved in the production of phytoalexin (Rustérucci *et al*., 1996; Mithöfer *et al*., 1997), the activation of calcium channels (Blume *et al*., 2000), MAPK cascades (Pitzschke and Hirt, 2006), systemic acquired resistance (SAR), hypersensitive response (HR) (Melillo *et al*., 2006; Mittler *et al*., 1999), senescence and programmed cell death (PCD) (Hung and Kao, 2004; Hung *et al*., 2006), as well as in stomatal closure (Pei *et al*., 2000).

1.3.3 Activation of MAPK cascades

Because of the immobility of plants, they have to adapt to the adversity by adjusting their own metabolism. Mitogen-activated protein kinase (MAPK) cascades can be activated by signals, such as microbial pathogen infection, injury, low temperature, drought, salt and reactive oxygen species (Widmann *et al*., 1999; Dangl and Jones, 2001; Yang *et al*., 2001; Zhang and Klessig, 2001). The MAPK pathway is highly conserved in eukaryotes, including three elements: MAPK kinase kinases (MAPKKK), MAPK kinases (MAPKK), and MAPK through a directional and sequential phosphorylation (Nürnberger *et al*., 2004), which can build a connection between the signal receptor upstream and response effectors downstream of the pathway.

Previous studies have shown that flg22 is a resistance-related elicitor in different plant cells (Meindl *et al*., 2000). After flg22 treatment, the phosphorylation of protein kinase FLS2 in *Arabidopsis* was shown to be necessary for signal transmission (Peck *et al*. 2001; Gómez-Gómez *et al*., 2001). Also the bacterial elicitor Harpin also was shown to induce

mitogen-activated protein kinase activity and the accumulation of transcripts for the pathogenesis-related protein HIN1 in tobacco (Lee *et al*., 2001). Furthermore, it has been demonstrated that different elicitors can induce common MAPK responses (Holley *et al*., 2003) indicating that this pathway acts as common element in defence signalling.

1.3.4 Plant hormones as stress mediators

Hormones are modulators of plant immunity (Pieterse *et al*., 2009; 2012). They are involved in various complicated networks, through which they modulate responses to different biotic and abiotic stresses. Especially jasmonic acid (JA), salicylic acid (SA), ethylene (Et) and auxin have been analysed in detail for their role in the regulation of plant defence (Thomma *et al*., 2001; Kazan and Manners, 2009; Pieterse *et al*., 2009). Jasmonic acid (JA) and its derivatives (Browse, 2009), and salicylic acid (SA) are thought to be directly involved in stress signalling (Vlot *et al*., 2009).

JA is an oxygenated fatty acid (oxylipin) involved in the resistance to necrotrophic pathogens, herbivores, as well as the wounding response (Bostock, 2005; Howe and Jander, 2008). SA is a phenolic compound synthesized by plants in response to a wide range of pathogens. The signalling mediated by SA includes the accumulation of reactive oxygen species (ROS) and pathogenesis-related (PR) proteins, the induction of the hypersensitive response (HR), callose deposition, and programmed cell death (PCD) (Baebler *et al*., 2014; Dinesh-Kumar *et al*., 2000). SA is also responsible for the activation of systemic acquired resistance (SAR) (Glazebrook, 2005; Grant and Lamb, 2006).

It has been reported that defence signalling between JA and SA is mutually antagonistic (Pieterse *et al*., 2012; Spoel *et al*., 2003). Several molecular mechanisms have been proposed to convey the antagonism between SA and JA signalling (Pieterse *et al*., 2012). The biological function of this antagonistic interaction might be to optimize the balance between defence and growth by optimal allocation of energy resources (Jaillais and Chory, 2010).

When the elicitors are recognized by plants, they activate a series of signalling events,

such as Ca^{2+} influx, ROS production, kinase activation, the generation of JA and SA, and changes in transcripts. These defence mechanisms proceed in a few minutes and then, within a few hours, plants can synthesize defence molecules, e.g. PR proteins and phytoalexins. These resistant reactions will kill pathogens or contain the damage to a limited region to protect plants (Samuel and Ellis, 2002; Liu *et al*., 2003).

1.4 Pathogen defence in grapevine

1.4.1 Sustainable viticulture

Grapevine is an important cash crop, but highly susceptible to diseases, what means high costs for plant protection. Viticulture accounts for 70% of the European fungicide consumption. The public acceptance for the massive use of fungicides is dwindling - the consumer wants to have poured "pure wine". To reduce chemicals, more environmentally friendly approaches are warranted. An important element for sustainable viticulture is resistance breeding using resistance factors from North American grapes (Gessler *et al*., 2011).

The economically important grapevine pathogens Downy and Powdery Mildew have evolved over millions of years together with wild American grapes, such that these wild grapes had enough time to evolve ETI and therefore can cope with these pathogens. However, these wild grapes are not suited for winery, due to their unpleasant "foxy taste" (off-flavour). As strategy to separate the desired immunity from the undesired flavour, those wild grapes have been crossed with cultivated grape varieties. In the progeny, one has to identify individuals that have inherited the resistance from the wild parent, whereas their good taste has to come from the cultivated parent. This strategy has been successful and culminated in economically important new varieties with good resistance against Downy Mildew (*Plasmopara viticola*). However, the rising success of these new varieties (for instance "Regent") is expected to initiate the next round of evolutionary warfare. In fact, the resistance conferred by the genetic factor "Resistance to *P. viticola* 3 (Rpv3)" which forms the base of most current disease-resistant grapevine varieties, has already been observed to become eroded by new strains of *Plasmopara* (Peressotti *et al*., 2010;

Gessler *et al*., 2011). As strategy to render the success of resistance breeding more sustainable, we need new sources of resistance.

1.4.2 The European Wild Grape *V. vinifera* L. *ssp. sylvestris*

Crop wild relatives (CWRs) have shifted into the centre of the attention of plant breeding and evolutionary biology (Ellstrand *et al*., 2010), because they represent valuable genetic resources for breeding. The cultivated grape *V. vinifera* L. *ssp. vinifera* has played an important role with respect to economy and culture over many centuries. It represents one of the most important crops worldwide considering its global distribution and its high economic value. However, its ancestor and CWR species, the European Wild Grape *V. vinifera* L. *ssp. sylvestris* Hegi is close to extinction. In order to conserve this species *ex-situ*, an extensive collection of the European Wild Grape (termed as *V. sylvestris* for the sake of simplicity) has been established (Nick, 2012) representing a complete copy of the genetic variation still present in Germany. A closer analysis of this collection revealed that many genotypes show good tolerance against several grapevine diseases, such as Downy Mildew (*Plasmopara viticola*), Powdery Mildew (*Erysiphe necator*), and Black Rot (*Guignardia bidwelli*), which were all introduced only 150 years ago from North America (Tisch *et al.*, 2014). From the two levels of plant innate immunity, ETI has evolved during a long arms race between pathogen and host plant. Since *V. sylvestris* is a naive host lacking a history of coevolution with these pathogens, it should not harbour any ETI-like defence against these diseases in contrast to North American wild species of *Vitis*. Therefore, the fact that genotypes of *V. sylvestris* can withstand these diseases might mean that they command a more efficient basal immunity (PTI) and have potential as new breeding resources to be exploited for sustainable viticulture in the future.

1.4.3 Stilbenes in grapevine

Stilbenes are a small family of plant secondary metabolites derived from the phenylpropanoid pathway, which are found in a limited number of plant species (Langcake and Pryce, 1976; Kodan *et al.*, 2001; Yu *et al.*, 2005). In the *Vitaceae*, stilbenes are important phytoalexins, which accumulate in response to various biotic and abiotic stresses such as pathogen attack (Langcake and Pryce, 1976; Adrian *et al*., 1997; Schnee

et al., 2008), UV-C irradiation (Bais *et al*., 2000), application of chemicals like aluminium ions and ozone (Rosemann *et al*., 1991, Adrian *et al*., 1996), or salinity stress (Ismail *et al.*, 2012). They also can be induced in response to plant hormones, such as jasmonates and ethylene (D'Onofrio *et al*., 2009; Belhadj *et al*., 2008a, 2008b). In grapevine, especially the stilbene *tran*s-resveratrol (*trans*-3,5,4′,-trihydroxy-*trans*-stilbene) has attracted particular attention, not only because of its antimicrobial activity, but also due to its possible pharmacological benefits to humans. The relatively low incidence of coronary disease in France despite a diet rich in saturated fatty acids (popularized as "French Paradox") has been attributed to regular uptake of resveratrol associated with mild consumption of red wine (Siemann and Creasy, 1992). Accumulating evidence indicates that this natural product can prevent some diseases, such as cardiovascular diseases, cancers, obesity, diabetes and neurodegenerative diseases, and in addition can cause an extension of lifespan (Baur and Sinclair, 2006; Roupe *et al*, 2006).

1.4.3.1 Stilbene biosynthesis

Plant stilbenes are derived from the general phenylpropanoid pathway (as shown in Fig.2). All higher plants are able to accumulate compounds like p-coumaroyl-CoA and cinnamoyl-CoA through the first important enzymes during this pathway, such as phenylalanine ammonia lyase (PAL), cinnamate-4-hydroxylase (C4H) and 4-coumarate: CoA ligase (4CL) (Ehlting *et al*., 2006; Ferrer *et al*., 1999; MacDonald and D'Cunha, 2007). The very essential branch in this pathway, initated by stilbene synthase as key enzyme, has only evolved in a limited number of plant species which therefore have acquired the capacity to produce stilbenes. Apart from sorghum (*Sorghum bicolor*) (Yu *et al*., 2005), for which only one stilbene synthase has been identified, in most plant species, e.g. peanut (Schröder *et al*., 1988), grapevine (Melchior and Kindl, 1991; Wiese *et al*., 1994), Scots pine (Preisig-Müller *et al*., 1999), and Japanese red pine (*Pinus densiflora*) (Kodan *et al*., 2002) stilbene synthases form large multigene families (Jaillon *et al*., 2007; Velasco *et al*., 2007; Richter *et al*., 2006). Chalcone synthase, the enzyme catalysing the first step of the pathway competing with stilbene biosynthesis, is the most ubiquitous type III polyketide synthases (PKSs) in plants and catalyses the first committed step of

flavonoid biosynthesis. Stilbene synthases compete with chalcone synthases for the substrates. Stilbene synthase show 75-90% amino acid identity with chalcone synthase and have arisen from a gene duplication followed by independent evolution of *StSy* and *CHS* genes (Tropf *et al*., 1994).

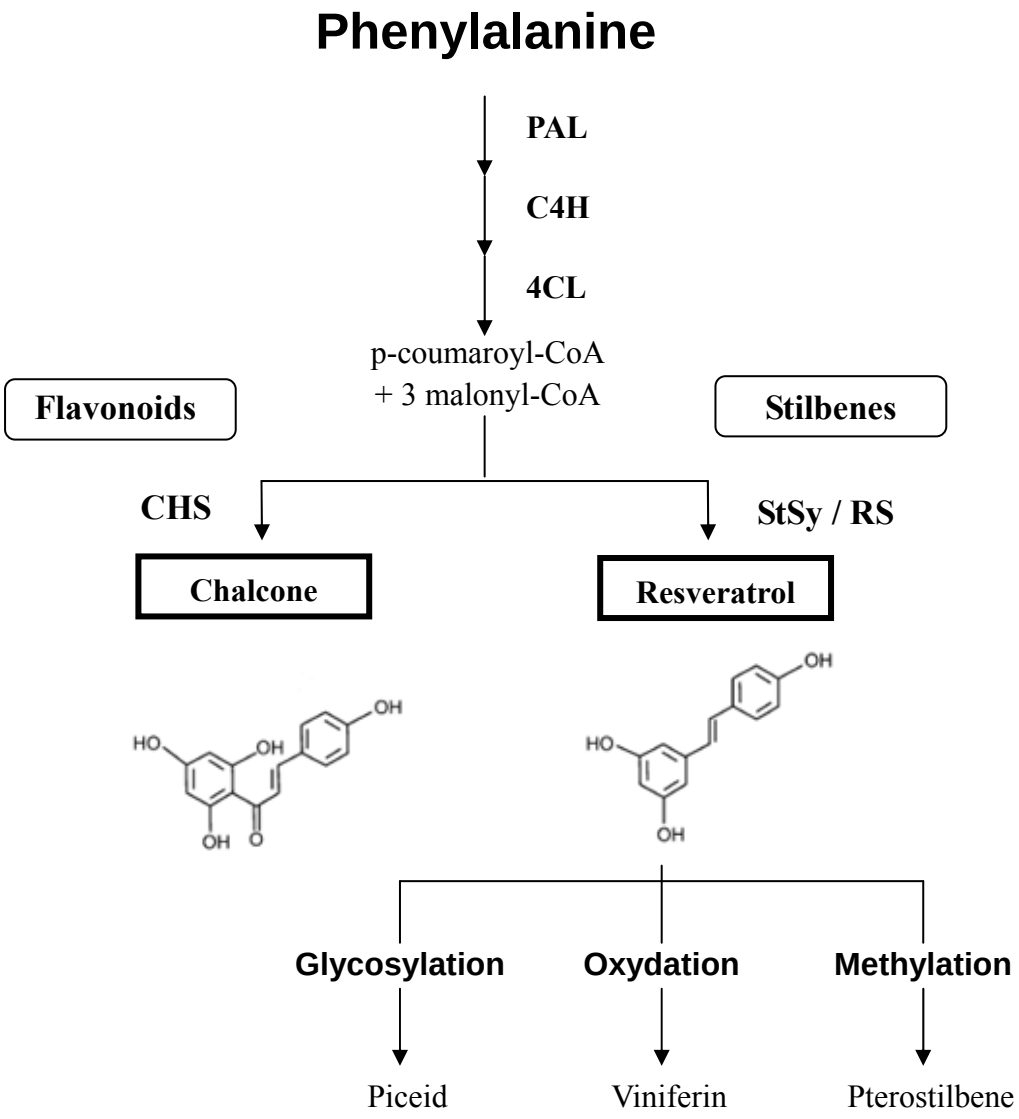


Fig.2 Simplified representation of the phenylpropanoid pathway with the positions of phenylalanine ammonium lyase (*PAL*), stilbene synthase (*StSy*), resveratrol synthase (*RS*), and chalcone synthase (*CHS*).

1.4.3.2 The biological roles of plant stilbenes

After biosynthesis, stilbenes can undergo several modifications, such as glycosylation to produce piceid, oxidation to produce viniferin and methylation to produce pterostilbene.

Glycosylation is a common modification of plant secondary metabolites (Gachon *et al*., 2005). The stilbene glucosides *trans*- and *cis*-piceid accumulate in healthy berries of different grape varieties (Gatto *et al*., 2008) and also represent the major stilbenes in commercial grape juices (Romero-Pérez *et al*., 1999). In plants, glycosylated stilbenes are generally existing with the free aglycons. Glycosylation of stilbenes could be function in their storage (Morales *et al*., 1998) and protect plants cells from potentially toxic effects of stilbenes (Hipskind and Paiva, 2000). The methylation form, pterostilbene, has caused great attention recently, not only because of its antimicrobial but also due to its pharmacological benefits to human, such as anti-cancer and anti-diabetic properties (Roupe *et al*., 2006). Resveratrol *O*-methyltransferase (ROMT) was found to catalyze resveratrol methylation to produce pterostilbene (Schmidlin *et al*., 2008). Pterostilbene accumulated in response to a variety of stresses (Langcake and McCarthy, 1979). The oxidation of resveratrol leads to viniferins (Jeandet *et al*., 2002), which can accumulate in grapevine leaves after the infection and UV irradiation (Langcake and Pryce, 1977).

Secondary metabolites play a key role in plant protection against environmental stresses (Dixon and Paiva, 1995). Plant stilbenes have been known to inhibit bacterial and fungal growth and therefore are considered as phytoalexins (Morales *et al*., 2000; Jeandet *et al*., 2002). Resveratrol inhibits conidial germination of *B. cinerea* (Adrian *et al*., 1997) and also could reduce the germination of sporangia of *P. viticola* (Pezet *et al*., 2004). Pterostilbene has a 5-fold higher activity than resveratrol in inhibiting fungal growth in *vitro* (Pezet *et al*., 2004) and also harbours toxicity towards fungal cells (Pezet and Pont, 1995). The antifungal activity of viniferin on *B. cinerea* conidia germination was very similar to that of pterostilbene (Pezet *et al*., 2004). The role of stilbenes in the resistance of grapevine to different pathogens has shown a correlation between stilbene accumulation in the toxic forms resveratrol and viniferin in resistant genotypes, whereas the susceptible genotypes tend to accumulate the non-toxic glycosylated piceid (Pezet *et al*., 2004; Schnee *et al*., 2008).

These antimicrobial activities of plant stilbenes strongly indicate that this part of secondary metabolism represents a very essential component of defence and may be used

as indicator of disease resistance.

1.4.3.3 Signalling pathways regulating stilbene biosynthesis

Signalling pathways regulating stilbene biosynthesis in plants cell are not fully understood and extremely complex. Some studies are performed on grapevine cell lines have shown that different elicitors could induce different outputs of stilbenes (Qiao *et al*, 2010; Chang *et al*., 2011; Chang and Nick, 2012), and in this process, several plant secondary messengers, especially calcium ions, reactive oxygen species, plant hormones have been identified to be involved in the regulation of stilbene accumulation.

Treatment of grapevine cells with various elicitors rapidly triggers Ca^{2+} influx, alkalinisation of the extracellular medium, ROS, and activation of MAP kinases. These early signals are generally followed by the induction of some defence related genes such as *PAL* and *StSy*, resulting in the accumulation of resveratrol and viniferins. (Chang *et al*., 2011; Chang and Nick, 2012; Aziz *et al*., 2003; Poinssot *et al*., 2003; Vandelle *et al*., 2006). Several studies also report the stimulation of stilbene accumulation by the application of exogenous methyljasmonate (MeJA) in grapevine suspension cells (Belhadj *et al*., 2008b; Krisa *et al*., 1999; Tassoni *et al*., 2005), accompanied by induction of *PAL* and *StSy* both at transcript and protein levels. A similar induction could be triggered by ethylene in grapevine leaves (Belhadj *et al*., 2008a) and peanut (Chung *et al*., 2001).

1.4.3.4 The transcription factors *MYB14* and *MYB15* regulate stilbene biosynthesis in grapevine

Until now, an increasing number of transcription factors (TFs) have been found to be responsible for the regulation of phenylpropanoid biosynthesis in several plants (Czemmel *et al*., 2012). In grapevine, several *MYB* TFs, such as the *MYB5a* and *MYB5b* were found to regulate the flavonoid pathway (Deluc *et al*., 2006, 2008). *MYBPA1* regulates the expression of the flavonoid pathway, leading to the production of proanthocyanidins (Bogs *et al*., 2007), and the grape *MYBF1* was shown to regulate the transcription of flavanol aglycone biosynthetic gene *FLAVONOL SYNTHASE1* (*FLS1*)

(Czemmel *et al*., 2009). *MYBA1* and *MYBA2* modulate *UDP-Glc: FLAVONOID–3–0-GLUCOSYLTRANSFERASE (UFGT)*, which encodes an enzyme responsible for the conversion of anthocyanidins to anthocyanins (Kobayashi *et al*., 2002; Walker *et al*., 2007). Matus *et al*. (2008) and Hichri *et al*. (2010) have shown that grapevine possesses more than 108 R2R3-MYB TFs. Besides *MYBF1*, all of the *MYB* TFs shown to regulate the flavonoid biosynthetic pathway in grapevine belong to the R2R3-MYB subgroup.

Höll *et al*. (2013) identified and functionally characterized two R2R3-MYB-type transcription factors (TFs) from grapevine, which regulate the stilbene biosynthetic pathway. These two TFs, named *MYB14* and *MYB15*, were found to be involved in the transcriptional regulation of stilbene biosynthesis. Höll *et al*. (2013) have shown, in transient gene reporter assays, *MYB14* and *MYB15* were confirmed to specifically activate the promoters of *StSy* genes.

1.5 The scope of this study

Stilbenes, as important phytoalexins, represent a central element of basal immunity. The aim of this work is to characterize the diversity of this *V. sylvestris* collection with respect to its capacity for stilbene biosynthesis, which might be exploited as a genetic resource for resistance breeding. We therefore screened *V. sylvestris* as ancestral species for genotypical differences in stilbene accumulation (stilbene “chemovars”). Since the response to pathogens is subject to considerable variation and dependent on seasonal influences, a short pulse of UV-C light was used as a well controllable trigger.

The transcriptional regulation of the stilbene biosynthetic pathway is mediated by two R2R3-MYB-type transcription factors, *MYB14* and *MYB15,* which were shown to activate the stilbene synthase promotor (Höll *et al.*, 2013). In this study, we test the idea whether the strong inducibility of stilbene synthase transcripts found in some *V. sylvestris* genotypes might result from elevated induction of *MYB14* and *MYB15*. Using a promotor-reporter assay in grapevine suspension cells (Höll *et al.*, 2013), we mapped known signalling events for PTI, such as dependence on NADPH oxidase, or induction

by jasmonic acid and calcium influx in order to find out whether the differences in the inducibility of the *MYB14* promotor can account for the stress-specific differences in the expression of stilbene synthase observed between representative genotypes.

Central questions were:

1. Is there genetic diversity of stilbene metabolism detectable in *V.sylvestris*?

In this study, we probed for potential genetic variation in *V. sylvestris* with respect to their output of stilbenes and potential use for resistance breeding. We have identified considerable variation in stilbene inducibility in *V. sylvestris*. We show genotypic differences in abundance and profiles of stilbenes that are induced in response to a UV-C pulse. Two clusters of stilbene "chemovars" emerged: one cluster with quick and strong accumulation of stilbenes, almost exclusively in form of the non-glycosylated forms resveratrol and viniferin. The second cluster accumulated less stilbenes and relatively high proportions of the glycosylated piceid. We further observed that the genotypic differences in stilbene accumulation were preceded by differential accumulation of the transcripts for stilbene-related genes: phenylalanine ammonium lyase (*PAL*), stilbene synthase (*StSy*), resveratrol synthase (*RS*), and chalcone synthase (*CHS*). A screen of the population with respect to susceptibility to downy mildew of grapevine (*Plasmopara viticola*) revealed considerable variability. The subpopulation of genotypes with high stilbene inducibility was significantly less susceptible as compared to low-stilbene genotypes, and for representative genotypes we could show that the inducibility of stilbene synthase to UV correlated with the inducibility by the pathogen.

2. Might the strong inducibility of stilbene synthase transcripts in some of these genotypes result from elevated induction of *MYB14*?

In our study, the *V. sylvestris* genotypes Hoe29 and Ke83 were found to be endowed with an elevated stilbene inducibility in response to UV light, which was correlated with a strong induction of stilbene synthase transcripts. Interestingly, in response to inoculation with *P. viticola*, stilbene synthase transcripts were elevated for Hoe29, but not for Ke83. Therefore, we tested the idea, whether the strong inducibility of stilbene synthase

transcripts in Hoe29 might result from elevated induction of *MYB14* and *MYB15*. Specific differences were discovered by next-generation sequencing and confirmed by cloning for the *MYB14* promotor of Hoe29, whereas *MYB15* did not reveal obvious changes. Both *sylvestris* genotypes share certain *MYB14* promotor domains (whereas they differ in others), which were absent from the promotor of the cultivated variety Augster Weiss (that is a weak stilbene producer). Using a promotor-reporter assay in grapevine suspension cells (Höll *et al.*, 2013), we show in the current work that differences in the inducibility of the *MYB14* promotor can account for the stress-specific differences in the expression of stilbene synthase observed between the three genotypes (Hoe29, Ke83, Augster Weiss). Although both *sylvestris* genotypes show good UV inducibility of *MYB14*, only the Hoe29 allele of this promotor is competent for induction by PTI (triggered by flg22). We further mapped known signalling events for PTI, such as dependence on NADPH oxidase, or induction by jasmonic acid and calcium influx. Our data suggest that a particular region in the promotor of a specific *sylvestris* allele of *MYB14* harbours potential as candidate target for resistance breeding.

2. Materials and Methods

2.1 Plant material

The *Vitis vinifera ssp. sylvestris* plants used in this study were collected (as cuttings) from natural sites at the 'Ketsch' peninsula at the Rhine River, in Southern Germany, which harbours the largest natural population in Central Europe (these accessions are indicated by “Ke”). Additionally, 25 *V. sylvestris* individuals originating from different sites in the Upper Rhine Valley (from the Hördt peninsula, indicated by “Hoe”) were included in this study. The details about the collection sites are described (Ledesma-Krist *et al.*, 2014) as well as 6 *V. vinifera* cultivars common in German and French vineyards (Augster Weiss, Pinot Blanc, Pinot Noir, Müller-Thurgau, Chardonnay, Cabernet Sauvignon), along with one American (*V. rupestris*), and one Chinese (*V. quinquangularis*) species. All accessions are maintained as living specimens in the grapevine collection of the Botanical Garden of the Karlsruhe Institute of Technology, and have been photographically documented, re-determined using morphological keys and ampelographic descriptors of the *Organisation Internationale de la Vigne et du Vin* (Olmo, 1976). For stilbene analysis, leaves of vineyard-grown plants were used over two subsequent years (2012 and 2013). For RNA extraction, the leaves were harvested from greenhouse-grown plants cultivated at a temperature of 22°C and 18°C (day and night, respectively) and a photoperiod of 14 h light to 10 h dark.

2.2 Preparation of leaf samples

To obtain fully expanded leaves of uniform size and comparable developmental state, the fourth and fifth leaves, counted from the apex, were excised from randomly selected individuals of the respective genotype, subjected to UV-C stress as described below and incubated upside down on moist filter paper in large Petri dishes. For the UV-C treatment the abaxial surface of the entire leaf was exposed to UV-C light (254 nm, 15 W, Germicidal, General Electric, Japan) for 10 min at a distance of 12.5 cm. The leaves of the different genotypes were harvested at different time points after the treatment,

immediately frozen in liquid nitrogen, and stored at -80°C until stilbene extraction and RNA analysis, respectively.

2.3 Stilbene extraction

To test, whether UV-C can induce stilbenes in a stable and reliable manner, leaves of all accessions were collected at indicated time points: C (control fresh leaf, without UV-C treatment), 0 (just at the end of the 10-min UV-C pulse), 3, 6, 24, 48 and 72 h, respectively, immediately frozen in liquid nitrogen, and stored at -80°C until further analysis. The frozen tissue was ground in liquid nitrogen using a pestle and mortar. An amount of 300 mg fresh weight of powdered leaf tissue were mixed with 1 ml of 100% methanol and homogenized for 10 min on a platform vortexer in order to maximize uniform sampling and to ensure complete extraction of the stilbenes. The homogenized samples were then centrifuged at 10 000 rpm for 10 min (Heraeus Biofuge Pico, Osterode, Germany). Before analysis, the supernatant was filtered by a disposable syringe filter (Pore size: 0.2 µm, Filter-Ø: 15 mm; Macherey-Nagel, Düren, Germany). All the experiments were performed under green safelight (λ_{max} 550 nm).

2.4 Stilbene analysis and quantification

For the initial experiments, the stilbenes extracted from *V. rupestris* and *V. quinquangularis* were analysed using high performance liquid chromatography (HPLC; Agilent 1200 series, Waldbronn, Germany) as described previously (Chang *et al.*, 2011) with minor modifications as follows: The mobile phases included acetonitrile (ACN), methanol and water in the following gradient: 2 min ACN/water (10/90 v/v); 15 min ACN/water (40/60 v/v); 20 min ACN/methanol/water (43/17/40 v/v/v); 23 min ACN/methanol (50/50 v/v); 25 min ACN/methanol (5/95 v/v); 35 min ACN/methanol (5/95 v/v); 39 min ACN/water (10/90 v/v); 42 min ACN/water (10/90 v/v). *Trans*-resveratrol was quantified and identified using an external standard on the basis of retention time and UV-VIS spectra. The standard for *trans*-resveratrol (Sigma-Aldrich, Deisenhofen, Germany) was dissolved in methanol at a concentration of 100 mg/L. Calibration curves used for quantification of the samples using this standard were strictly

linear (r^2 > 0.99). At least three independent biological replicates from subsequent seasons were conducted.

To extend the analysis to the numerous cultivars of *V. sylvestris* and *V. vinifera*, liquid chromatography-mass spectrometry (LC-MS) analyses were performed at the metabolomics platform of the Institut National de Recherche Agriculturel (INRA, Université de Strasbourg, Colmar, France) after comparative studies with the same samples had shown that the results between the methods were identical. The analysis method was as follows: Acetonitrile and formic acid of liquid chromatography-mass spectrometry grade were supplied by Thermo Fisher; water was provided by a Millipore water purification system. Methanolic leaf extracts were analysed using an UHPLC system (Dionex Ultimate 3000, Thermo Fisher Scientific) equipped with a binary pump, an online degasser, a thermostated autosampler, a thermostatically controlled column compartment, and a diode array detector (DAD). Chromatographic separations were performed on a Nucleodur C18 HTec column (50 x 2 mm, 1.8 µm particle size; Macherey-Nagel, Düren, Germany) maintained at 20°C. The mobile phase consisted of acetonitrile/formic acid (0.1%, v/v) (eluent A) and water/ formic acid (0.1%, v/v) (eluent B) at a flow rate of 0.40 mL/min. The gradient elution program was as follows: 0 to 1 min, 85% B; 1 to 6 min, 85% to 5% B; 6 to 7 min, 5% to 85% B; and 7 to 8 min, 85% B. The sample volume injected was 1 µL. The liquid chromatography system was coupled to an Exactive Orbitrap mass spectrometer (Thermo Fischer Scientific, San Jose, USA) equipped with an electrospray ionization source operating in the negative mode. Parameters were set to 300°C for ion transfer capillary temperature, and 2500 V for needle voltage. Nebulization with nitrogen sheath gas and auxiliary gas were maintained at 50 and 5 arbitrary units, respectively. The spectra were acquired within the m/z mass range of 100 to 1000 atomic mass units (a.m.u.), using a resolution of 50,000 at m/z 200 a.m.u. The system was calibrated externally using the Thermo Fischer calibration mixture in the range of m/z 100-2000 a.m.u., giving a mass accuracy better than 2 ppm. Stilbenes were identified according to their mass spectra, UV absorption spectra and retention times, and compared to those of authentic standards. The instruments were controlled using the

XCalibur software, and data were processed using the XCMS software (Smith *et al*., 2006). Stilbene quantifications were based on calibration curves obtained with the respective standards. Trans-piceid, trans-resveratrol, and trans-pterostilbene standards were purchased from Sigma-Aldrich (L'Isle d'Abeau, France). (+)-ε-viniferin and (+)-δ-viniferin standards were purchased from Polyphenols Biotech (Villenave d'Ornon, France). Cis forms of stilbenes were obtained by photo isomerization under UV light of trans-stilbene standard solutions. Solutions containing of 0, 5, 10, 25, 50 and 100 µg/ml of the standards were used for calibrations, with good linearity ($r^2 > 0.95$). Three independent biological replicates from subsequent seasons were conducted and all analyses were repeated twice.

2.5 RNA extraction and cDNA synthesis

The leaves of Augster Weiss, Hoe29, Ke53 and Ke83 were harvested at 0, 0.5, 1, 3, 6 and 24 h after irradiation as well as for non-treated controls.

For controlled inoculation with downy mildew (*Plasmopara viticola*) a suspension of 40 000 sporangia/ml was used as given in detail below, when the screening is described. To circumvent potential modulation of gene expression by a wounding response, this experiment was not conducted with leaf discs, but with entire leaves. The controlled inoculation leaves of Augster Weiss, Hoe29 and Ke83 were harvested at C (control fresh leaf), 120 h-C (control leaf incubated in the absence of *Plasmopara viticola* under the same conditions), and 120 h-S (the leaf was infected with *Plasmopara viticola* – suspension and incubated for 120 h), respectively, immediately frozen in liquid nitrogen and stored at -80°C until RNA extraction.

Total RNA was isolated using Spectrum^{TM} Plant Total RNA Kit (Sigma, Deisenhofen) according to the protocol of the manufacturer. The extracted RNA was treated with a DNA-free DNase (Qiagen, Hilden, Germany) to remove potential contamination of genomic DNA. The mRNA was transcribed into cDNA using the M-MuLV cDNA Synthesis Kit (New England Biolabs; Frankfurt am Main, Germany) according to the instructions of the manufacturer. The RNase inhibitor (New England Biolabs; Frankfurt

am Main, Germany) was used to protect RNA from degradation. The amount of RNA template was 1μg.

2.6 Semi-quantitative RT-PCR

Semi-quantitative reverse transcription-PCR (RT-PCR) was performed following 30 cycles of 30 s denaturation at 94 °C, 30 s annealing at 60 °C, and 1 min synthesis at 68 °C in a conventional PCR cycler (Biometra, Göttingen, Germany) as described previously (Qiao *et al*. 2010; Chang *et al*., 2011; Chang and Nick, 2012), using the primers given as following and the detailed information in the Table 1:

Table 1 Primers list and literature references used for semi-quantitative RT-PCR and quantitative real-time PCR for this study.

Name	GenBank accession no.	Primer sequence 5'-3'	Reference
EF1-α	EC959059	Sense: 5'- -3' TGTCATGTTGTGTCGTGTCCT Antisense: 5'- -3' CCAAAATATCCGGAGTAAAAGA	This paper
PAL	X75967	Sense: 5'- -3' TGCTGACTGGTGAAAAGGTG Antisense: 5'- -3' CGTTCCAAGCACTGAGACAA	Belhadj *et al*. (2008b)
RS	AF274281	Sense: 5'- -3' TGGAAGCAACTAGGCATGTG Antisense: 5'- -3' GTGGCTTTTTCCCCCTTTAG	This paper
StSy	X76892	Sense: 5'- -3' CCCAATGTGCCCACTTTAAT Antisense: 5'- -3' CTGGGTGAGCAATCCAAAAT	This paper
CHS	AB066274	Sense: 5'- -3' GGTGCTCCACAGTGTGTCTACT Antisense: 5'- -3' TACCAACAAGAGAAGGGGAAAA	Belhadj *et al*. (2008b)
MYB14	ABW34392	Sense: 5'- -3' GGGGTTGAAGAAAGGTCCAT Antisense: 5'- -3' GGCCTCAGATAATTCGTCCA	This paper

(Notes: *EF1-α*, elongation factor 1α; *PAL*, phenylalanine ammonia lyase; *RS*, resveratrol synthase; *StSy*, stilbene synthase; *CHS*, chalcone synthase; *MYB14*, the transcription factor of *StSy*.)

The PCR reaction was performed with Taq Polymerase from New England Biolabs (NEB, Frankfurt, Germany) and ThermoPol buffer (NEB). The PCR products were separated by conventional 2% agarose gel electrophoresis after visualization with SybrSafe (Invitrogen, Darmstadt, Germany). Images of the gels were recorded on a MITSUBISHI P91D screen (Invitrogen) using a digital image acquisition system (SafeImage, Intas, Germany). Each experiment was repeated for at least three biological replicates.

2.7 Quantitative Real-Time RT-PCR

Quantitative real-time RT-PCR was performed on the Opticon 2 system (Biorad, München) as described (Svyatyna *et al*., 2014). After denaturation at 95 °C for 3 min, 40 cycles followed (95 °C for 15 s and 61 °C for 40s). Data were exported from the Opticon cycler and imported into the Opticon Monitor (Biorad, München). Quantitative real-time RT-PCR analysis was carried out in 20 µL reactions containing in final concentration 200 nM of each primer, 200 nM of each dNTP, 1 x GoTaq colourless buffer, 2.5 mM additional $MgCl_2$, 0.5 U GoTaq polymerase (Promega, Mannheim, Germany), 1 x SYBR green I (Invitrogen, Darmstadt, Germany) and 1 µL of the cDNA template in a 1:10 dilution. Three technical replicates were performed for each sample. C_t values obtained were processed for plotting a graph using an Excel spreadsheet. To compare the mRNA expression level among different samples, the C_t values from each sample were normalized to the value for *EF-1α* as internal standard obtained from the same sample. This internal standard is widely used in studies on stilbenes due to its stability and reliability (Reid *et al*., 2006; Polesani *et al*., 2008) and also found to be very stable in our previous work under different biotic and abiotic stress conditions (Qiao *et al*. 2010; Chang *et al*., 2011; Chang and Nick, 2012; Ismail *et al.*, 2012). Since in our hands, actin, which is often used as housekeeping reference, did not show any deviations from *EF-1α* (Gong and Nick, unpublished), we decided to calibrate expression data on this internal standard. For each triplicate, these normalized C_t values were averaged. The difference between the C_t values of the target gene X and those for the *EF-1α* reference R were calculated as follows: $\triangle C_t\,(X) = C_t\,(X) - C_t\,(R)$. The final result was expressed as $2^{-\triangle Ct\,(X)}$.

2.8 Principal Component Analysis and statistical evaluation of metabolomic and genetic data

Principal component analysis (PCA) was performed using the princomp command functioning under R (R Core Team, 2013) using the following argument (cor=T, scores=T). The contribution of the stilbenes to the construction of the axis of the PCA was obtained using R software and the methodology described at

http://www.R-project.org/. To infer phylogenetic relationships, DNA was extracted from leaf tissue by a slightly modified CTAB method (Doyle and Doyle, 1987) using about 25 mg leaf tissue shock-frozen in liquid nitrogen and homogenised. Samples were genotyped at 9 microsatellite loci located on different chromosomes (http://www.genres.de/eccdb/vitis/) using the SSR-markers: VVS2 (Thomas and Scott, 1993), VVMD07 (Bowers *et al.*, 1996), VVMD25, VVMD27, VVMD28, VVMD32 (Bowers *et al.*, 1999), VrZag62, and VrZag79 (Sefc *et al.*, 1999), the phylogenetic relationship was inferred using the UPGMA method (Sneath and Sokal, 1973) using the software MEGA4 (Tamura *et al*., 2007) with default settings.

2.9 Screening *V. sylvestris* for susceptibility to downy mildew

To screen differences in the susceptibility of the European Wild Grape (*V. sylvestris*) accession to downy mildew (*Plasmopara viticola*) at least seven leaf discs taken from the 4th to 5th fully expanded leaf of each genotype cultivated in the greenhouse were transferred in a randomised manner to Petri dishes containing 5 ml sterile tap water, inoculated with one droplet of a spore suspension (30 µl for each leaf disc, 40.000 sporangia/ml), which was removed 24 h post-inoculation (hpi), and incubated in a climate chamber at high humidity and 21°C (day-night cycle 12 h : 12 h). Sporulation was first evaluated visually according to Kortekamp (2006) and Genet *et al*. (1997) at 7 days post-inoculation (dpi). In addition, production of spores was scored: each leaf disc was transferred to a 1.5 ml tube and complemented with 1 ml of 0.1% v/v Tween-80 in distilled water. The tube was vigorously shaken (Vortex) to achieve a homogenous suspension, and the concentration of sporangia was determined using a hematocytometer (Fuchs-Rosenthal). The data are means obtained from at least two different years. For all experiments, an isolate was used that is routinely maintained on Müller-Thurgau in the greenhouse of the State Education and Research Center Rheinpfalz.

2.10 Determination of stomatal density

To evaluate stomatal density, glue imprints of fully expanded healthy fresh leaves, harvested from plants grown in the green house of the Botanical Garden of the KIT were

used. Glue imprints were obtained using the lower, abaxial, leaf surfaces of four different leaves of each accession as template. A drop of glue (UHU Hart Modellbaukleber 45510, UHU GmbH & Co. KG, Bühl, Germany) was placed on the respective leaf region near the leaf base. To allow for high-quality imaging, intercostal fields with a sufficiently planar surface were used in the region between midrib and lateral vein, and covered by a thin and homogenous layer, distributing the glue with the finger tip. After 5-10 minutes, the glue has cured to a thin film, conserving an imprint of the leaf surface. This imprint could then be removed using a pair of tweezers and placed on an object slide in a drop of distilled water. Gray-scale microscopical images were collected from these glue imprints with differential interference contrast (DIC) using a digital imaging system (Zeiss Axio Scope, equipped with a CCD-camera AxioCam). Pictures were recorded at 20 times magnification with 2720 x 2048 pixel and saved as RGB color tif file for evaluation with ImageJ. All stomata and epidermal cells on the picture were quantified using the plugin Analyze – Cell Counter. Stomata density was defined as ratio of the stomata of one picture per epidermal cells of the same picture, a parameter that was found to be independent of leaf expansion, leaf differentiation, and year (Table 4). Between 200 and 600 stomata were scored along with epidermal pavement cells to determine stomata density. Values represent medians from four independent samples collected over two subsequent vegetation periods.

2.11 Cloning the promoter constructs of *MYB14* and *MYB15*

2.11.1 TA cloning and Gateway®-Cloning for *MYB14*

In order to get the full length of Hoe29, Ke83 and Augster Weiss including promoter regions, the genomic DNA was extracted according to the protocol in Appendix 5.1 and amplified according to the reference sequence in the GenBank (NW_003724037.1) with the specified primers in Appendix 5.2. The PCR was performed in Appendix 5.3 and Phusion™ DNA polymerase (NEB, Frankfort, Germany) was used. The PCR products were excised from the gel and purified using the NucleoSpin® Extract ǁ (Macherey-Nagel, Dueren, Germany) kit according to the manufacturer instructions. A-overhangs were added to the PCR product by A-tailing (purified PCR product 8.75 µl; Taq Polymerase

0.05 µl; 10 x ThermoPol buffer 1 µl; 10 mM dNTPs 0.2 µl; total volume 10 µl) at 72 °C for 1 h, and the A-tailing product was directly inserted into vector pGEM-T® Easy (Promega Corporation, USA). The ligation protocol was shown in Appendix 5.5.1 and confirmed by DNA sequencing (GATC Biotech, Cologne, Germany). The confirmed sequences were compared with the database sequence using the Multiple sequence alignment program ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). After the alignments, the specific gateway primers (Appendix 5.2) were designed to amplify the chosen promoter sequence of Hoe29 (1351 bp), Ke83 (1245 bp) and Augster Weiss (1347 bp) from the pGEM-T® Easy vector using the Gateway®-Cloning technology (Invitrogen Corporation, Paisley, UK). The PCR was performed by Q5 High-Fidelity DNA Polymerase (NEB, Frankfort, Germany) in Appendix 5.4. The promoter regions of these three genotypes were ligated into luciferase vector pLuc (Horstmann *et al.*, 2004) and confirmed by DNA sequencing (GATC Biotech, Cologne, Germany). The promoter sequences of Hoe29, Ke83 and Augster Weiss were shown in Appendix 5.7.1. The TA-cloning and the Gateway®-Cloning technology overview were shown in Appendix 5.5 and 5.6.

2.11.2 Cloning the promoter sequence of *MYB15*

In order to get the promoter sequence of Hoe29, Ke83 and Augster Weiss, the genomic DNA was amplified according to the reference sequence in the GenBank (NW_003724021.1) with the designed primers: sense: 5'-3' GCCAAGGACTTGACTTGGAA, antisense: 5'-3' TGTTCTCCAATAGGAACACCAC. The PCR was performed with Phusion™ DNA polymerase (NEB, Frankfort, Germany) as described above in Appendix 5.3. The PCR products were excised from the gel and purified using the NucleoSpin® Extract Ⅱ (Macherey-Nagel, Dueren, Germany) kit according to the manufacturer instructions. A-overhangs were added to the PCR product by A-tailing (described as above) at 72 °C for 1 h, and the A-tailing product was directly inserted into vector pGEM-T® Easy (Promega Corporation, USA) and confirmed by DNA sequencing (GATC Biotech, Cologne, Germany). The confirmed sequences were compared with the database sequence using the Multiple sequence alignment program

ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The *MYB15* promoter sequences were shown in Appendix 5.7.2.

2.12 Suspension cultures and medium

A suspension culture was established from the callus tissue of petioles from *V. vinifera* L. cv. 'Chardonnay'. The cell culture was subcultured weekly by adding 10 ml culture into the log-Phase fresh of 50 ml Grape Cormier (GC) medium (Bao Do and Cormier, 1991). The suspension culture was continuously shaken at 90 rpm, 25 °C in the dark condition.

In Vitro, plants were applied on Chee and Pool (C2D) *Vitis* medium (Duchefa, Haarlem Niederlande) including macro- and micronutrients as described previously (Chée and Pool, 1987). Grape Cormier Medium was prepared as follows: 3.2 g/L Gamborgs B5 with minimal organics (Sigma G5893), 30 g/L Sucrose, 0.25 g/L Casein Hydrolysate, 0.93 µM Kinetin, 0.54 µM NAA, pH 5.8 (with 1 N KOH). For GC agar plates were 0.8% plant-agar (Duchefa) added to the medium.

2.13 Transient expression experiments and dual luciferase assay

2.13.1 Microcarrier preparation

In a 1.5 ml microfuge tube, weighed out 60 mg of microparticles (Bio-Rad); added 1 ml of 70% ethanol, freshly prepared; vortexed on a platform vortexer for 5 minutes; incubated for 15 minutes; pelleted the microparticles by spinning for 5 seconds in a microfuge; removed the liquid and discarded; repeated the following steps three times: (I) added 1 ml of sterile water, (II) vortexed for 1 minute, (III) allowed the particles to settle for 1 minute, (IV) pelleted the microparticles by spinning for 2 seconds in a microfuge, (V) removed the liquid and discarded; added sterile 50% glycerol 1ml to bring the microparticle concentration to 60 mg/ml (assumed no loss during preparation); stored the microparticles at room temperature for up to 2 weeks/4 °C for up to 36 months.

2.13.2 Coating DNA onto microcarriers

When removing aliquots of microcarriers, it was important to vortex the tube containing the microcarriers continuously in order to maximize uniform sampling. Vortexed the

microcarriers prepared in 50% glycerol (60 mg/ml) for 5 minutes on a platform vortexer to resuspend and disrupt agglomerated particles; removed 50 µl (3 mg) of microcarriers to a 1.5 ml microfuge tube; while vortexing vigorously, added in order: (I) 50 µl $CaCl_2$ (2.5 M), (II) 20 µl spermidine (0.1 M); continued vortexing for 2-3 minutes; allowed the microcarriers to settle for 1 minute; pelleted the microcarriers by spinning for 2 seconds in a microfuge; removed the liquid and discarded; added 140 µl of 70% ethanol without disturbing the pellet; removed the liquid and discarded; added 140 µl of 100% ethanol without disturbing the pellet for 2 times; removed the liquid and discarded; added 50 µl of 100% ethanol; gently resuspended the pellet by tapping the side of the tube several times, and then by vortexing at low speed for 2-3 seconds; removed 3 x 16 µl aliquots of microcarriers and transferred them to the center of a macrocarrier. An effort was made to remove equal amounts (1 mg) of microcarriers each time and to spread them evenly over the central 1 cm of macrocarrier using the pipette tip.

2.13.3 Bombardment

A transient expression system was performed using the 'Chardonnay' suspension cell culture as described in Bogs *et al*. 2007 and Walker *et al*. 2007. The cells were bombarded with 1.6 µm gold particles (Bio-Rad) at a distance of 9.5 cm using the gene gun Model PDS-1000/He Biolistic Particle Delivery System (Bio-Rad) with 4481 kPA helium pressure and a vacuum of 86 kPA. The mixed gold particles including the 50 ng of promoter DNA, 100 ng control plasmid pRluc (Horstmann *et al*., 2004). To ensure all the experiments at a comparable level, the total amount DNA was 2 µg in mixed gold particles, and empty vector (PART 7) was complemented if less than 2 µg. The bombarded cells were lysed after the bombardment 48 h using the dual luciferase assay in the follows steps.

2.13.4 Dual luciferase assay

A transient assay was performed as described previously (Czemmel *et al*., 2009; Merz *et al*., 2015) with minor modifications as follows: (I) Lysis of gene gun transformed cells: after a bombardment (48 h), the cells were harvested after a treatment with different stresses at a certain time (3 ~ 4 h), and then used a spatula to transfer (~200 µl) of the

central part of the cells (or part where you could see the gold particles) into a mortar; added 200 µl of 2 x Passive Lysis Buffer (PLB, Promega, Madison, Wl) and grinded for 1.5 min with a pestle on ice; centrifuged for 1 min at 10.000 rpm. (II) Luciferase activity measurement was performed with the dual-luciferase reporter assay system (PJK, Kleinblittersdorf, Germany) in two tubes: allowed samples, Beetle Juice and RenillaGlow Juice to reach room temperature; applied 2 x 20 µl of the lysate to two different polypropylene tubes; added 50 µl Beetle Juice and mixed 3 times to measure (firefly luciferase); discarded tube; added 50 µl RenillaGlow Juice to the second tube and mixed 3 times to measure (renilla luciferase). Light emission was measured with a lumat LB9507 Luminometer (Berthold Technologies, Bad Wildbad, Germany) and the relative luciferase activity was calculated as the ration between the firefly and Renilla (control) luciferase activity. All experiments were performed in triplicates with similar relative ratios to the respective control and all experiments repeated from three independent biological replicates.

2.14 Cell treatments for transient promoter assays

For the UV-C experiment, the cells were treated for 2 min at a distance of 12.5 cm by UV-C (15 W, OSRAMHNS, OFR) and then harvested at 3 h. The bacterial peptide flg22, a 22-amino-acid peptide, was purchased from a commercial producer (Antikörper online, Aachen, Germany), and diluted in sterile H_2O as a stock solution of 1 mM. Diphenyleneiodonium chloride (DPI) (Sigma-Aldrich, Germany) was dissolved in dimethylsulfoxide (DMSO) as a stock solution of 10 mM. Calcium ionophore (Sigma-Aldrich, Germany) was diluted in DMSO as a stock solution of 50 mM. Gadolinium chloride ($GdCl_3$) (Sigma-Aldrich, Germany) was used as inhibitor of mechanosensitive calcium channels and diluted with DMSO to a 20 mM stock solution. PD98059, a chemical inhibitor for the mitogen-activated protein kinase (MAPK) cascade (Sigma-Aldrich, Deisenhofen, Germany), was dissolved and sterilised into a 100 mM stock solution in DMSO. Salicylic acid (SA) (Sigma-Aldrich, Germany) and (±)-jasmonic acid (JA) (Sigma-Aldrich, Germany) was dissolved in ethanol (EtOH) to obtain the stock solution of 50 mM and 500 mM respectively. The inhibitor 1-phenylpyrazolidinone

(phenidone) (Sigma-Aldrich, Germany) was dissolved in DMSO to obtain a 2 M stock solution. Polyoxyethylene-20-sorbitan monolaurate (Tween® 20) was obtained from Carl Roth in Germany. All treatments were accompanied by appropriate solvent controls, and the maximal concentration of solvent used in the test samples did not exceed 1 ‰.

3. Results

3.1 Genetic diversity of stilbene metabolism in *Vitis sylvestris*

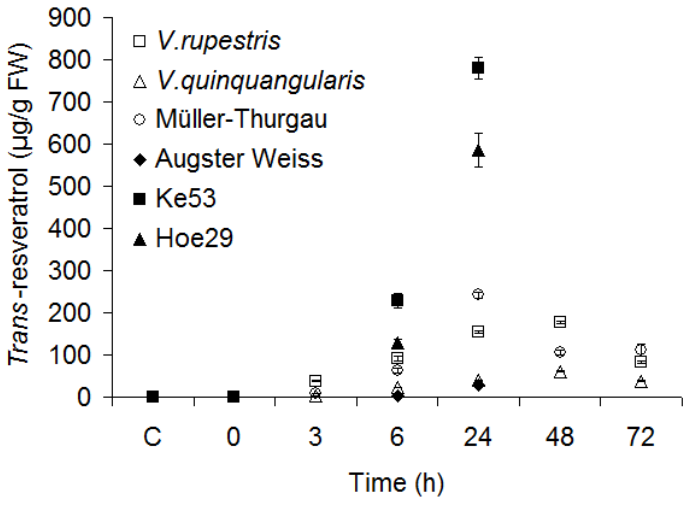
3.1.1 Stilbene accumulation can be triggered by UV-C

In order to compare stilbene inducibility in different genotypes, a trigger is required that is easy to standardize and can be applied to leaf tissue in a reliable manner. From preliminary studies testing different candidate triggers such as methyl jasmonate or inoculation with *P. viticola*, a short pulse of UV-C (10 min) was found to produce the most reliable results (Douillet-Breuil *et al*., 1999). We first followed the accumulation of *trans*-resveratrol over time in response to this UV-C pulse in representative genotypes using HPLC (Fig. 3A). As representative examples we show the data for two wild *non-vinifera* species (*V. rupestris*, a North American wild grape, and *V. quinquangularis*, a Chinese wild grape), two *vinifera* cultivars (‘Müller Thurgau’, a cultivar commonly grown in the Upper Rhine Region, and ‘Augster Weiss’, a male-sterile ancient variety, which is used for breeding), as well as two *sylvestris* genotypes, Hoe29, and Ke53, falling into different subclades of *sylvestris*. Prior to the treatment (control), and immediately after the pulse (defined as 0 h), the content of *trans*-resveratrol was below the detection limit in all genotypes. The abundance of *trans*-resveratrol increased from 3 h after UV-C irradiation, reaching a maximum from 24 h to 48 h, followed by a decline till 72 h. However, the amplitude of the response differed strongly between genotypes indicating that the accumulation was genotype dependent. For instance, around three times more resveratrol accumulated in *V. rupestris* compared to *V. quinquangularis*, whereas cultivar Müller-Thurgau accumulated more than cultivar Augster Weiss. However, these differences were minor compared to the strong accumulation found in the two *sylvestris* genotypes Hoe29 and Ke53. To compare stilbene accumulation between different genotypes, we used control, 0 h, 6 h, and 24 h as representative time points in the following experiments.

To visualize not only genotypic differences in the total abundance of stilbenes, but possibly differences in stilbene profiles, the levels of *trans*-piceid, *cis*-piceid,

trans-resveratrol, *cis*-resveratrol, ε-viniferin, δ-viniferin, pterostilbene, *trans*-piceatannol and *cis*-piceatannol were quantified in parallel for the different time points using LC-MS. As shown for a selection of representative genotypes in Fig. 3B and C, there was a great genotypic variation in stilbene inducibility. Whereas UV-C induced a quick and strong accumulation of stilbenes in the genotypes Pinot Blanc, Ke53, Ke83 and Hoe29 (Fig. 3B), the same treatment produced hardly any accumulation in the genotypes Augster Weiss, Ke89, Ke51, and Ke78 (Fig. 3C), even at 24 h. Combined analysis of all 86 genotypes (Fig. 4) showed that accumulation of piceid, resveratrol and piceatannol were observed already 6 h after UV-C exposure, whereas viniferins accumulated later and were mostly detected 24 h after exposure. This time-dependence in the stilbene pattern is shown in Fig. 3B for Pinot Blanc, Ke53, Ke83 and Hoe29. Here, the total stilbene content increased significantly from 6 h, which could be mainly attributed to the accumulation of *trans*-resveratrol, whereas at 24 h, resveratrol was complemented by viniferins. For example, in Ke53, 234 µg/g FW of resveratrol was measured at 6 h, with only low levels of viniferin (7 µg/g FW). In contrast, at 24 h, although the content of resveratrol had significantly increased, by more than threefold, to 818 µg/g FW, during the same time viniferin had increased even more, by more than 40-fold (333 µg/g FW). The total stilbene content was therefore 1230 µg/g FW and exceeded the UV-C induced stilbene accumulation in genotypes such as Ke89 by more than 25 times (For example, even at 24 h, the total stilbene content in Ke89 reached only 49 µg/g FW).

A



B **C**

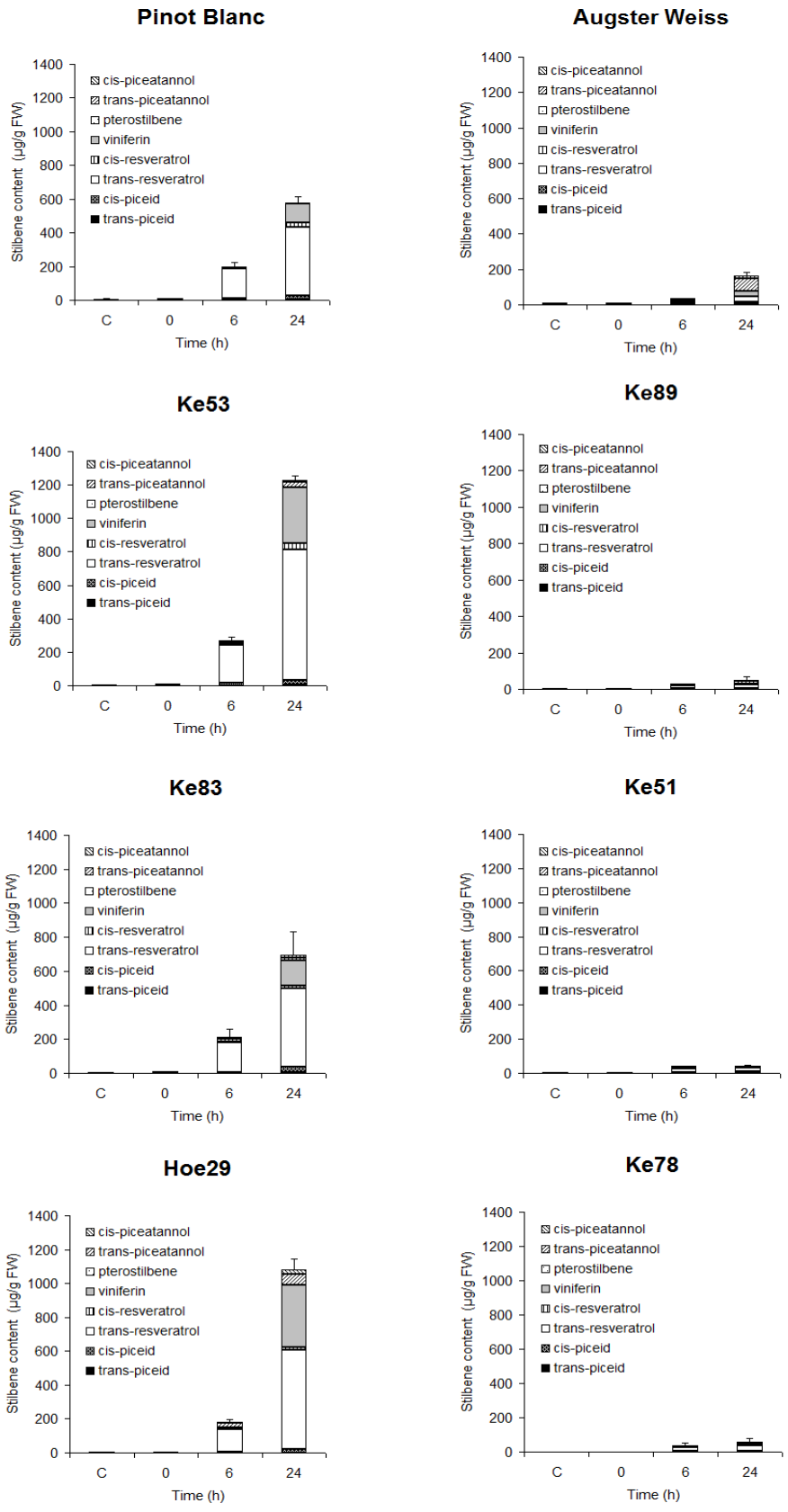

Pinot Blanc
Augster Weiss
Ke53
Ke89
Ke83
Ke51
Hoe29
Ke78
Stilbene content (µg/g FW)
Time (h)
C
0
6
24
cis-piceatannol
trans-piceatannol
pterostilbene
viniferin
cis-resveratrol
trans-resveratrol
cis-piceid
trans-piceid

Fig.3 Time courses of stilbene accumulation in different genotypes in response to UV-C. (A) Time courses for the accumulation of *trans*-resveratrol in *V. rupestris*, *V. quinquangularis*, Müller Thurgau, Augster Weiss, Ke53 and Hoe29. Representative time courses for strong stilbene accumulation in Pinot Blanc, Ke53, Ke83 and Hoe29 (B), and weak accumulation in Augster Weiss, Ke89, Ke51 and Ke78 (C). Data represent mean values and standard errors from three independent biological replicates.

3.1.2 Genetic variation of stilbene accumulation

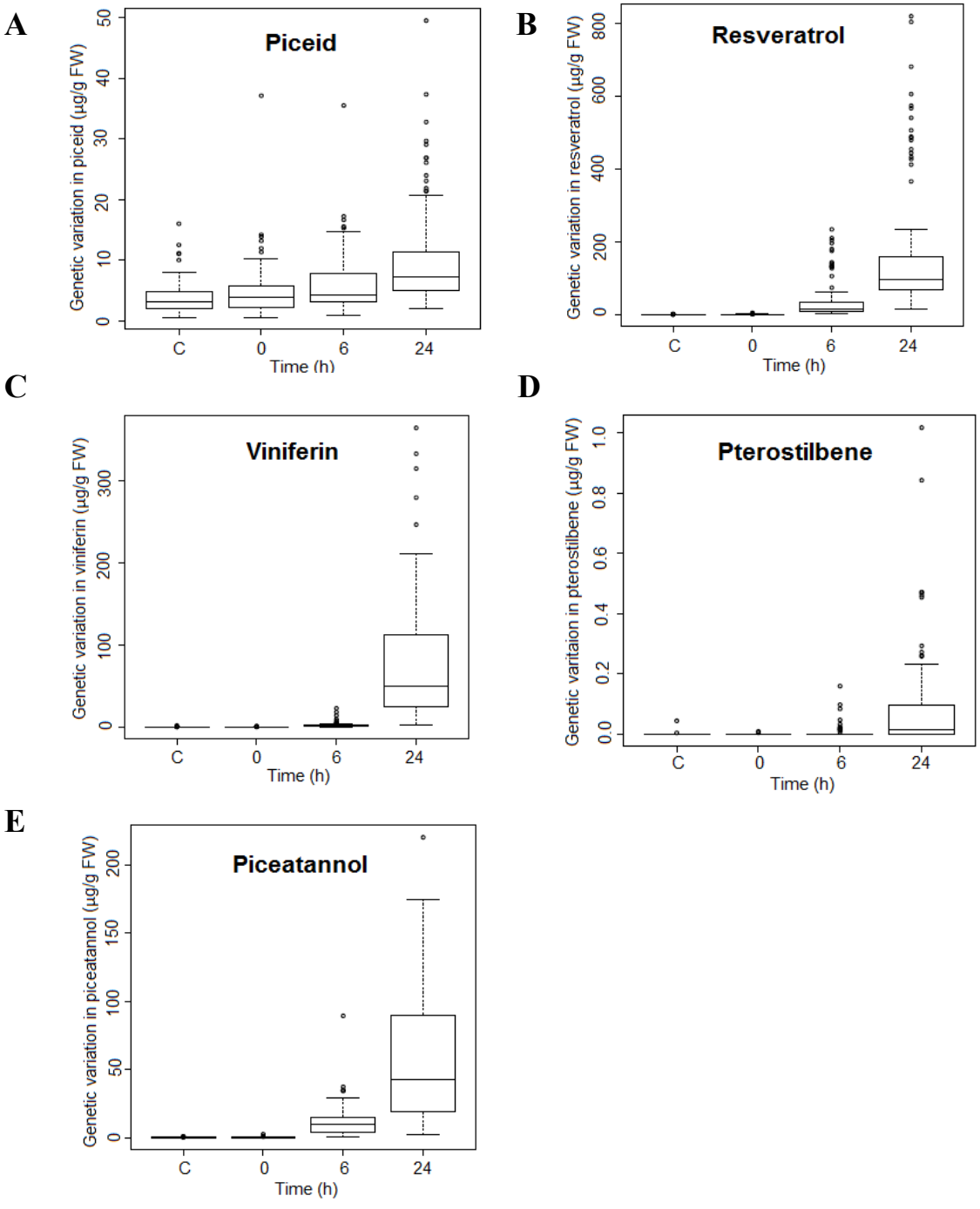


Fig.4 Genetic variation in the stilbene response to UV-C. The accumulation of different stilbene species was determined for 86 genotypes of *V. sylvestris* and a few cultivars. Values are represented in the boxplot format, whereby the box comprises the data for the central 50% of the sample, the horizontal solid line represents the median value, and the dotted line gives the position of the maximal and minimal values excluding the outliers, the outliers are indicated as individual points and were defined as those values that were beyond 1.5 times the upper or lower quartile, respectively. (A) Pooled *cis*- and *trans*-piceid; (B) pooled *cis*- and *trans*-resveratrol; (C) viniferin; (D) pterostilbene; (E) pooled *cis*- and *trans*-piceatannol.

In order to evaluate the extent of the genetic variation in defence metabolism present in *V. sylvestris*, we followed stilbene accumulation in 86 genotypes over time in response to UV-C. As shown in Fig. 4A-E, all analysed stilbenes (*cis*- and *trans*-piceid, *cis*- and *trans*-resveratrol, viniferins, pterostilbene and *cis*- and *trans*-piceatannol) accumulated significantly with increasing time. For piceid, resveratrol, and piceatannol, the increases were observed at early stages (from 6 h after UV-C exposure). In contrast, the accumulation of the downstream derivatives viniferins and pterostilbene occurred later: at 6 h, these two stilbene species were still not detectable, but had substantially increased at 24 h. In all genotypes, resveratrol and viniferins were the predominant stilbenes, and the abundance of viniferins and resveratrol were tightly correlated (the correlations between different types of stilbenes are given in Fig.5 and Table 2).

In the frame of these general patterns, there was considerable variation as represented by the width of the boxplot bars and the position of the outliers. In some genotypes, such as Pinot Noir, Pinot Blanc, Ke15, Ke20, Ke22, Ke28c, Ke39, Ke53, Ke83, Ke84, Ke95, Ke96, Ke99, Ke103, Hoe17 and Hoe29, much more resveratrol was produced than in the bulk of the populations (Fig. 4B, see the dots on the top of the boxplot at 24 h); among those, Ke28c, Ke39, Ke53, Ke84 and Hoe29, also accumulated much more viniferins compared to the bulk of the population (Fig. 4C, see the dots on the top of the boxplot at 24 h).

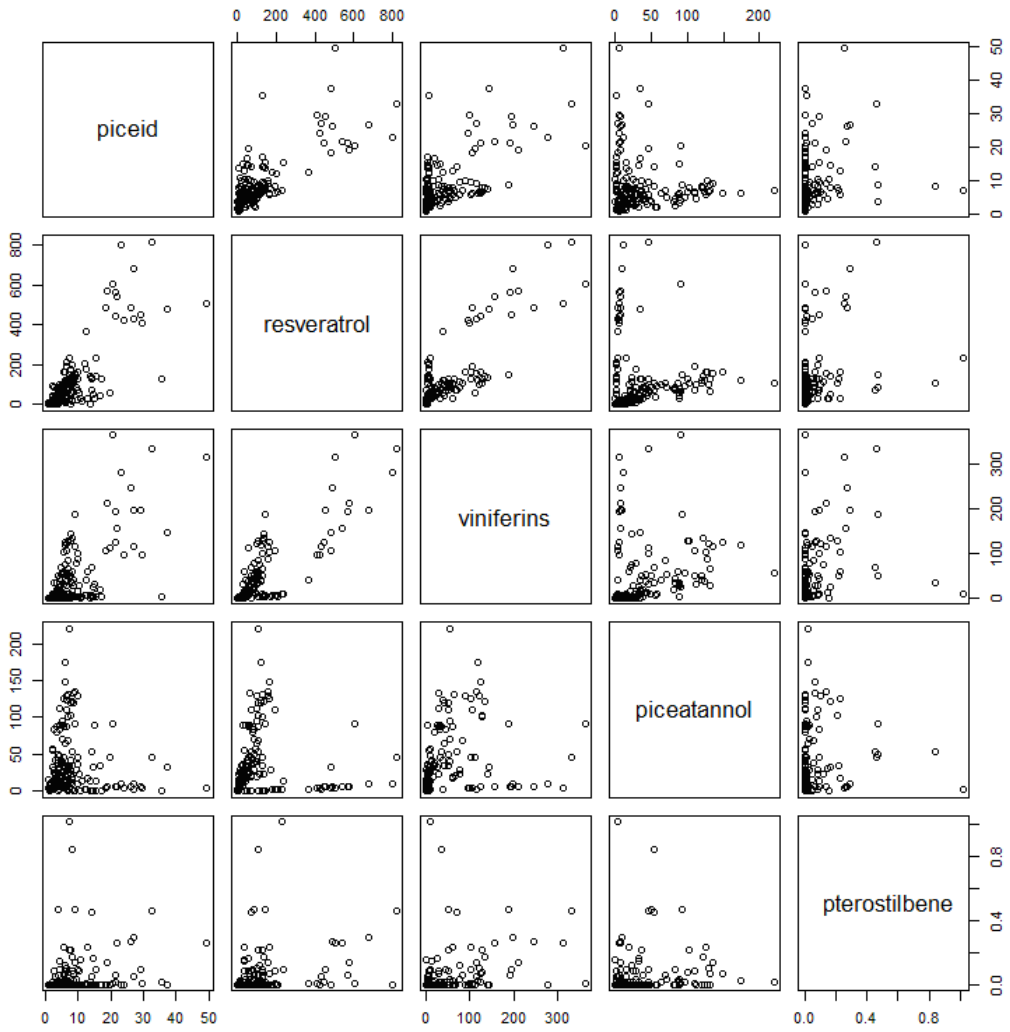


Fig.5 Correlations between the amounts of piceid, resveratrol, viniferins, piceatannol and pterostilbene.

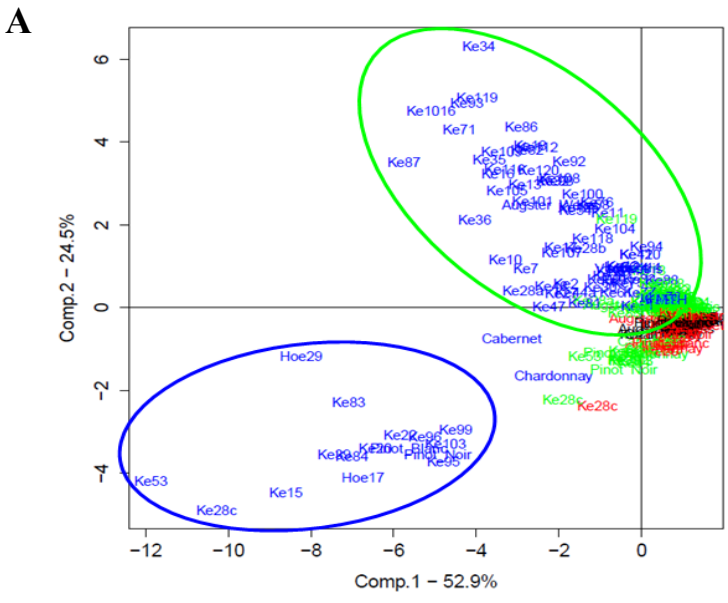
Table 2 Correlations between the amounts of piceid, resveratrol, viniferins, piceatannol and pterostilbene.

	piceid	resveratrol	viniferins	piceatannol	pterostilbene
piceid	1.00	0.81	0.74	-0.36	0.16
resveratrol	0.81	1.00	0.83	-0.31	0.18
viniferins	0.74	0.83	1.00	-0.05	0.20
piceatannol	-0.37	-0.31	-0.05	1.00	-0.11
pterostilbene	0.16	0.18	0.20	-0.11	1.00

3.1.3 Two types of stilbene "chemovars"

To understand the factors underlying stilbene variation in *V. sylvestris* (also in relation to some cultivars common in the Upper Rhine Valley and the two *non-vinifera* species from North America and China), the metabolomics data of all 86 genotypes for all time points were subjected to a Principle Component Analysis (PCA). As shown in Fig. 6A, the first two principal components could explain 77.4% of the variation between the samples (the contribution of each individual stilbene species to these two principal components is given in Table 3). Hereby, the amount of stilbenes (Comp. 1) accounted for 52.9% of the variation between the samples, which means that the variation present at 24 h could be mainly attributed to the overall content of stilbenes. In contrast to this quantitative trait, Comp. 2 was rather qualitative and based on the composition of the accumulating stilbenes. This explained 24.5% of the variation.

From the principal component analysis (PCA) at T=24 h, two clusters of genotypes emerged, which differed in both quantitative and qualitative parameters. The first (smaller) cluster is characterized by the strong ability to accumulate stilbenes, especially in the form of resveratrol and viniferins (Fig. 6A, blue circles). This cluster comprises Pinot Noir, Pinot Blanc, Ke15, Ke20, Ke22, Ke28c, Ke39, Ke53, Ke83, Ke84, Ke95, Ke96, Ke99, Ke103, Hoe17 and Hoe29. The second (larger) cluster comprises genotypes accumulating less stilbenes, which a relatively high proportion of piceid and piceatannol.



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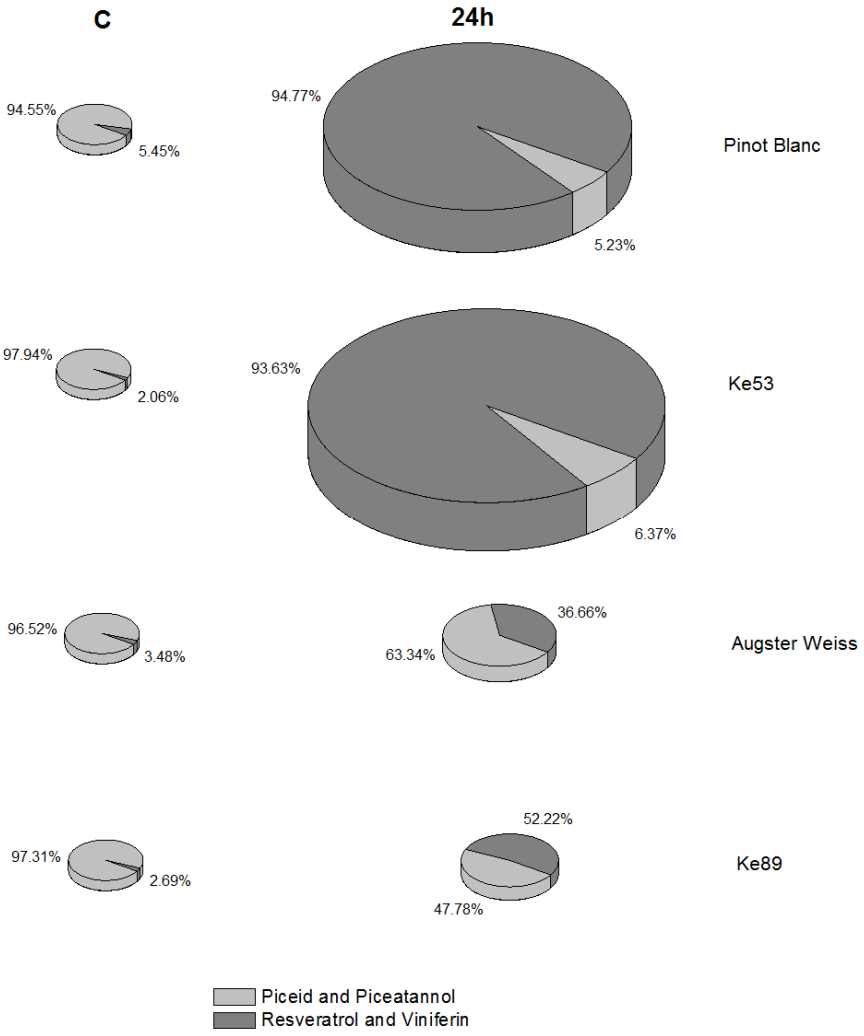


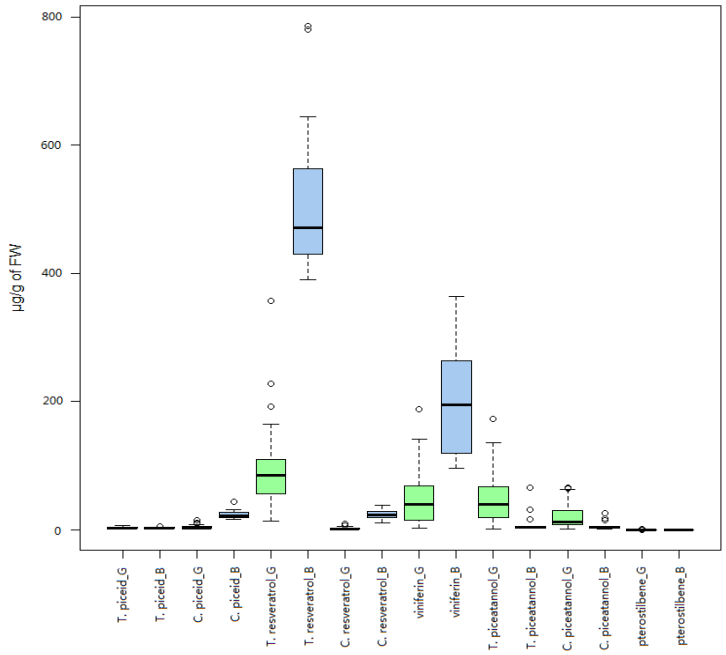
Fig.6 Two stilbene "chemovars" in *V. sylvestris*. (A) Principal Component Analysis (PCA) over time-dependent accumulation of different stilbene species in a population of 86 genotypes of grapevine. Black: controls (untreated fresh leaf); red (0 h), green (6 h), and blue (24 h) give different time points after a UV-C pulse of 10 min. The PCA comprises data from three independent experimental series measuring *cis*-piceid, *trans*-piceid, *cis*-resveratrol, *trans*-resveratrol, viniferin, *cis*-piceatannol, *trans*-piceatannol and pterostilbene. (B) Representative stilbene profiles of four genotypes. The relative proportion of piceid and piceatannol versus resveratrol and viniferin is shown for the control and 24 h after the UV-C pulse. The total abundance of stilbenes is represented by the size of the cake. Pinot Blanc, and Ke53 belong to the blue cluster shown in Fig. 6A, Augster Weiss and Ke89 belong to the green cluster.

Table 3 The construction of stilbenes for each component in principal component analysis.

	Comp.1	Comp.2
trans-piceid	-0.395	0.282
cis-piceid	-0.323	-0.395
trans-resveratrol	-0.433	-0.231
cis-resveratrol	-0.401	-0.339
viniferin	-0.449	
trans-piceatannol	-0.239	0.533
cis-piceatannol	-0.265	0.533
pterostilbene	-0.251	

To illustrate the conclusions from the PCA analysis that the genotypes cluster with respect to stilbene profile, two representative genotypes arbitrarily selected from each cluster are shown in Fig. 6B. Pinot Blanc and Ke53 belong to the blue (high-stilbene type) cluster, whereas Augster Weiss and Ke89 were chosen from the green (low-stilbene type) cluster. In the controls, the overall abundance of stilbenes was low (represented by the small size of the cake). Those stilbenes that can be detected are almost exclusively present as piceid – the glycosylated form of resveratrol (Fig. 6B). In response to the UV-C pulse, all genotypes accumulated the stilbene species resveratrol and its oxidized form, the viniferins. However, the genotypes from the green (low-stilbene type) cluster (Augster Weiss and Ke89) also accumulated some piceid and piceatannol, which at 24 h accounted for about 50% - 60% of total stilbenes, whereas in genotypes from the blue (high-stilbene type) cluster (Pinot Blanc and Ke53), piceid and piceatannol remained below 7%. When this difference between “blue” and “green” genotypes was tested statistically (Fig.7A and Fig.7B), the genotypes from blue cluster were found to contain significantly more resveratrol and viniferin compared to those from green one. In contrast, the green cluster contained a significant higher piceatannol/total stilbenes ratio.

A



B

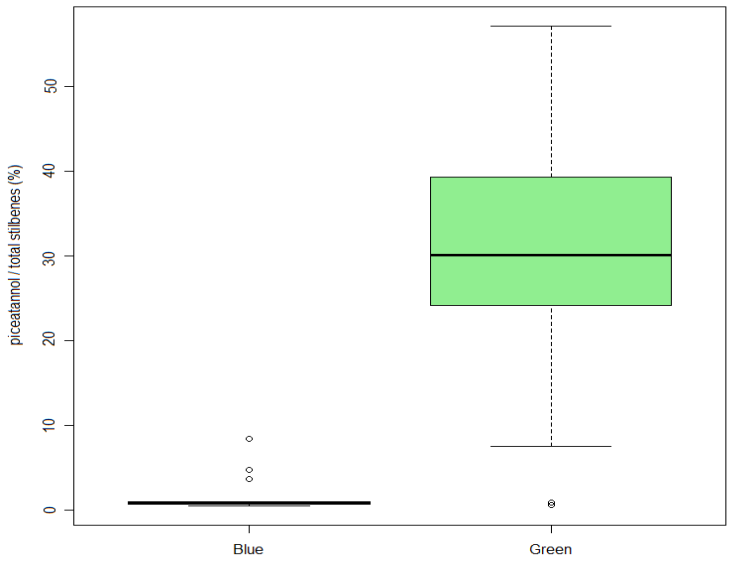


Fig.7 (A) Boxplots of the amounts of each stilbene in the blue (B) and in the green (G) cluster. The amounts of *trans*-piceid and pterostilbene are not statistically different in both clusters (t-test, *p*-val=0.6951 and 0.006 respectively), whereas, *cis*-piceid, *trans*-resveratrol, *cis*-resveratrol, viniferins, *trans*-piceatannol, *cis*-piceatannol are statistically differently accumulated in the blue cluster in comparison to the green one (t-test, *p*-val=5.51 x 10^{-9}, 3.1 x 10^{-10}, 1.77 x 10^{-9}, 5.37 x 10^{-6}, 4.71 x 10^{-8} and 7.48 x 10^{-7}, respectively). (B) Boxplots of the piceatannol/total stilbene ratio in the blue and green cluster. The ratio is statistically greater in the green cluster in comparison to the blue one (t-test, *p*-val<2.2 x 10^{-16}).

These data show that there exist two stilbene "chemovars" in *V. sylvestris*. The chemovars of the "blue" cluster accumulate high levels of stilbenes in non-glycosylated form, whereas the chemovars of the "green" cluster accumulate low levels of stilbenes, with a relatively high proportion of piceid and piceatannol.

3.1.4 Strong stilbene inducibility is distributed in specific clades of *Vitis sylvestris*

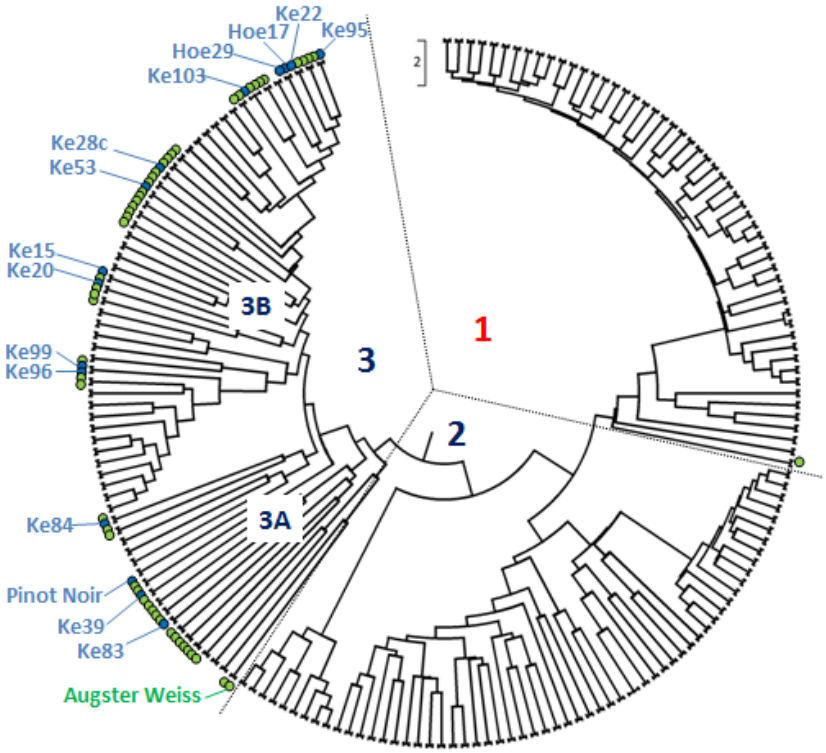


Fig.8 Genetic relationships for stilbene inducible genotypes of *V. sylvestris*. The incidence of genotypes from the green (piceid-rich chemovars) and the blue (viniferin-rich chemovars) clusters (as defined in Fig. 6A) were plotted into an UPGMA tree over nine SSR markers for 361 taxa of European *V. sylvestris*, *V.vinifera*, and American non-*vinifera*. Inferred by the UPGMA method. Tree drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. 1, non-*vinifera* genotypes; 2, *sylvestris* genotypes from outside of Central Europe; 3, German-Austrian *sylvestris*.

The genetic differences in stilbene inducibility represent an interesting genetic resource

for resistance breeding. We therefore wondered, whether the genotypes of the "blue" (high-stilbene type) cluster (Fig. 6A) are equally distributed over all genotypes from the Ketsch peninsula, or whether they are concentrated on specific clades. The phylogenetic relationship between these genotypes was inferred from microsatellite genotyping and integrated with published data for those microsatellites to comprise a set of 361 taxa of European *sylvestris*, *vinifera*, and non-European *Vitis* for these 9 SSR markers (Fig. 8, Ledesma-Krist *et al.*, 2014). These markers had been selected from the literature, because they are the most informative to resolve relationships in *sylvestris*. The topology of the tree was tested by Bayesian clustering, and found to remain very robust after including the first 6 markers (S. Schröder *et al.*, unpublished results). The accessions from the Ketsch peninsula formed a separate cluster together with *V. sylvestris* from the Upper Danube Valley and *V. vinifera* cultivars current in German vineyards, whereas the *V. sylvestris* accessions from Spain, the Rhône valley, and South East Europe formed a separate cluster, as well as the *non-vinifera* accessions that established a third cluster. When those genotypes that had been analysed with respect to their stilbene inducibility were mapped on this tree, the genotypes of the "blue" (high-stilbene type) cluster were found to be distributed non-homogenously. For instance, among the 15 genotypes, where both data sets (SSR markers and stilbene profiles) had been established, only four were found in subcluster 3A, whereas 11 were found in subcluster 3B, within subcluster 3B, five clustered into the right-most branch of the clade.

3.1.5 Piceid does not serve as a precursor for the biosynthesis of non-glycosylated stilbenes

Some genotypes accumulate relatively high levels of piceid (Fig. 4A, see the dots on the top of boxplots). The glycosylation of piceid protects against oxidation into oxidative dimers, such as viniferins and, therefore, piceid has been proposed to act as storage form for bioactive resveratrol and viniferins (Regev-Shoshani *et al*. 2003). We therefore asked, whether piceid might function as precursor for later release of resveratrol. To illustrate this exemplarily for the UV-C response, we selected the two strong piceid accumulators, Ke28c and Ke10, because these genotypes show comparable resting levels of piceid and

resveratrol/viniferin.

Both genotypes showed high basal levels of piceid compared with other genotypes (Fig. 4A). If these high basal levels of piceid were a storage form to produce the bioactive, non-glycosylated, stilbenes, Ke28c and Ke10 should show elevated induction of non-glycosylated stilbenes. However, when they were exposed to UV-C, these two genotypes produced completely different results with respect to stilbene accumulation. Although almost the same amounts of piceid (Fig. 9A) and total stilbenes (Fig. 9B) were measured in the controls, Ke10, while only slightly increasing the levels of piceid, induced around >20 times more non-glycosylated stilbenes as compared to the basal level. In contrast, Ke28c accumulated, upon UV-C induction, more than 3 times of piceid as compared to Ke10, but more than 6 times of non-glycosylated stilbenes as compared to Ke10.

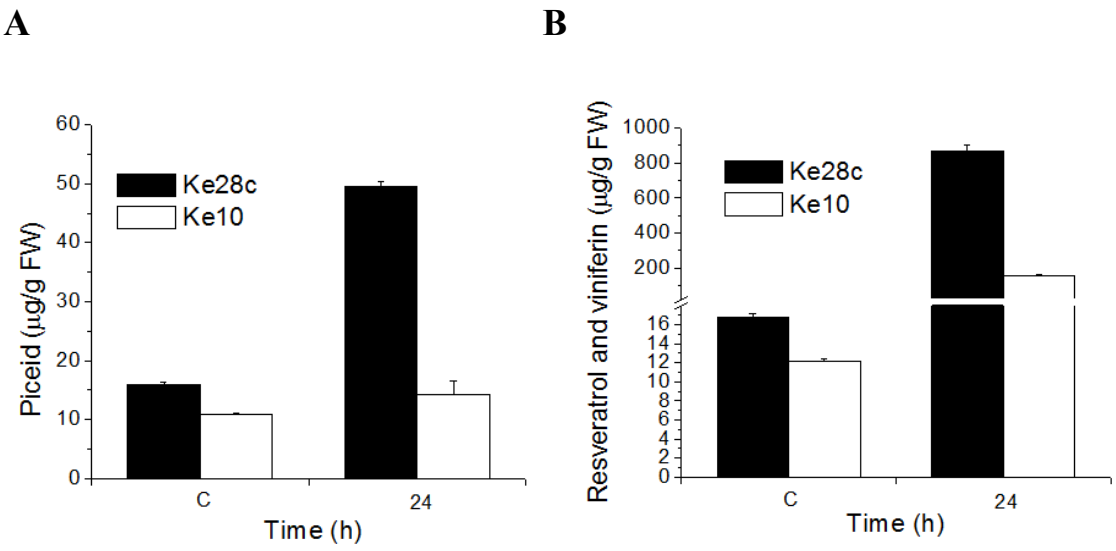


Fig.9 Variation in stilbene inducibility of piceid accumulators. Amounts of piceid (A) and non-glycosylated stilbenes (B) under control conditions and 24 h after a UV-C pulse in Ke28c and Ke10. Data represent mean values and standard error from three independent replicates.

So, we can conclude that some of the genotypes with higher basal levels of preformed piceid also produce more stilbenes upon induction, but some do not. Even in Ke10, the level of non-glycosylated stilbenes found at 24 h exceed the resting level of piceid by more than 20-fold, which means that the vast majority of induced bioactive stilbenes must

be synthesized *de novo* rather than being released from a glycosylated precursor. For the genotypes of the blue cluster, the high levels of resveratrol (Fig. 4B, the dots on the top of the boxplot at 24 h) that, in case of Ke39, Ke53, Ke84 and Hoe29 are accompanied by high amounts of viniferins (Fig. 4C, the dots on the top of the boxplot at 24 h), all show only very low resting levels of piceid in control conditions. This means that these genotypes produce their strong induction of stilbenes completely through *de novo* synthesis. Release of resveratrol from preformed piceid does not play any role in this induction. To directly follow the metabolic flow through stilbene formation, pulse labelling with radioactive precursors (such as phenylalanine) might be a strategy.

3.1.6 Response of stilbene-related genes to UV-C

To investigate whether the observed genotypic differences in stilbene accumulation can be correlated with a corresponding transcriptional response, we followed the transcript level of key genes in representative genotypes by semi-quantitative RT-PCR and quantitative real-time PCR. As shown by the simplified stilbene biosynthetic pathway in Fig. 2, the general activation of the phenylpropanoid pathway was monitored by probing phenylalanine ammonia-lyase (*PAL*), the stilbene branch of the pathway by probing for stilbene synthase (*StSy*), and resveratrol synthase (*RS*), whereas the competing flavonoid branch was by probed via chalcone synthase (*CHS*). Elongation factor 1α (*EF1-α*) was used as internal standard. It should be kept in mind that the stilbene synthase family in grapevine is extremely expanded with numerous members that are very similar, often even identical in their open reading frames, but differ with respect to their promotors (Parage *et al.*, 2012; Vannozzi *et al.*, 2012). The transcripts picked up by the *StSy* and *RS* oligonucleotide primers are therefore likely to stem from different members of this family, and differ partially in their expression patterns (e.g. Qiao *et al.* 2010). In the following, the operational denominators "*StSy*" and "*RS*" will be used. As strong stilbene accumulators we chose Hoe29, Ke53, and Ke83, as representative of three different phylogenetic clades of *V. sylvestris* (Fig. 8), whereas Augster Weiss (an ancient cultivar, which is male sterile and therefore often used for molecular breeding) was selected as representative for the weakly accumulating genotypes.

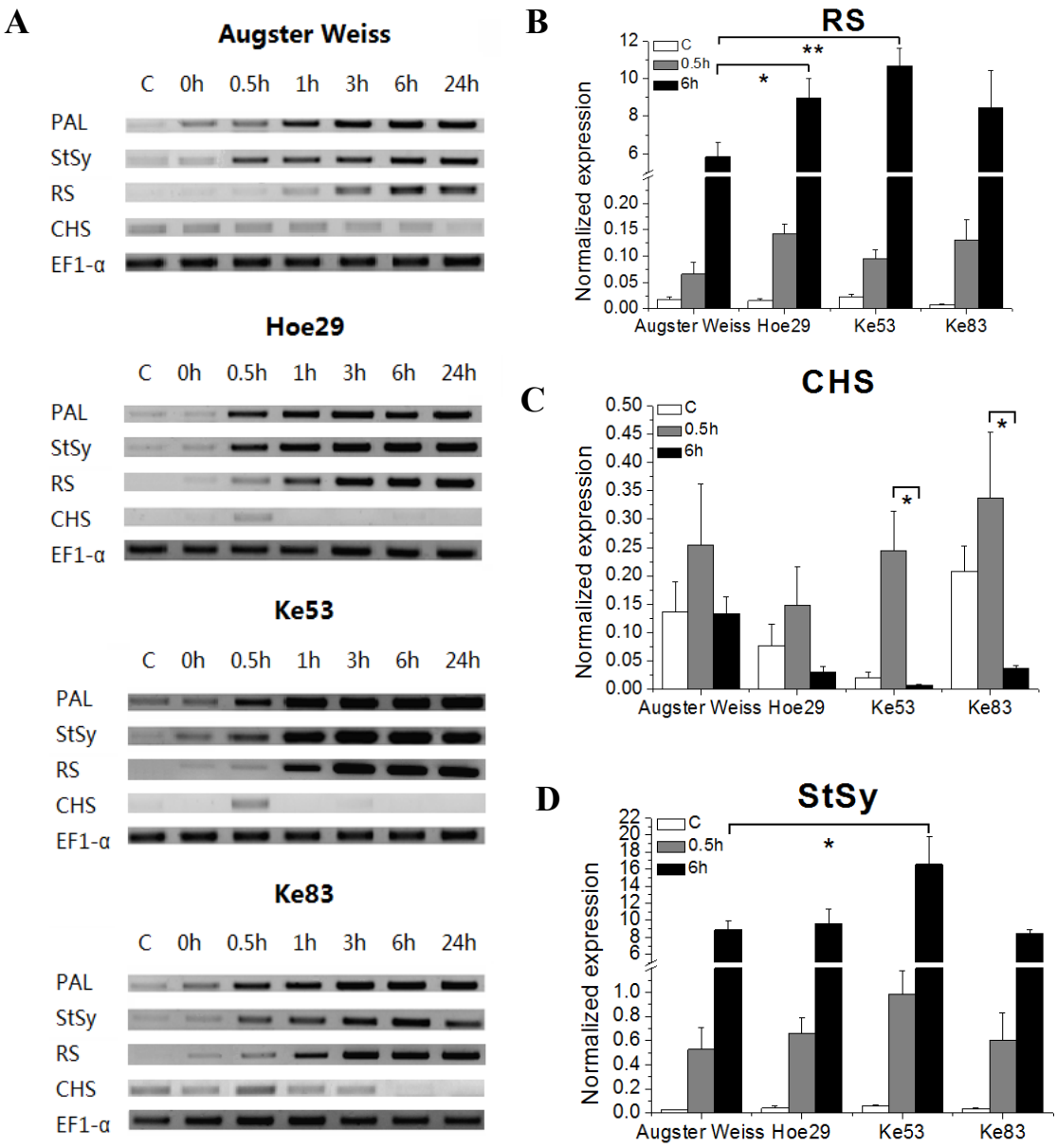


Fig.10 Time courses of the UV-C response for key genes of the phenylpropanoid pathway. (A) Representative agarose gels with the amplificates from semi-quantitative RT-PCR for untreated controls and different time points after irradiation with 10 min of UV-C compared to elongation factor *EF1-α* as internal standard. (B-D) Quantification of transcripts by quantitative real-time PCR normalized to the expression of elongation factor *EF1-α*. * indicate differences that are statistically significant on the $P < 0.05$ level and ** indicate $P < 0.01$ level. Data represent mean values from five independent experimental series, error bars represent standard errors.

As shown in Fig. 10A, hardly any transcripts could be detected for the controls and the time point just at the end of the 10 min UV-C pulse, irrespective of the genotype,

indicating that the basal steady-state levels of these genes are very low. In all strong stilbene accumulators, *PAL* transcripts were found to be induced already 30 min after the pulse treatment, whereas in Augster Weiss, the induction of *PAL* transcripts was delayed by 30 min and did not reach the same amplitude. The induction of *PAL* transcripts was accompanied by almost simultaneous induction of *StSy* transcripts, whereas *RS* transcripts followed 1-2 h later. Again, the response in Augster Weiss was delayed and less pronounced as compared to the strong stilbene accumulators. Interestingly, for Hoe29 and Ke83, the induction of *StSy* did not differ from Augster Weiss, indicating that different stilbene synthase genes can differ in their regulatory pattern (Fig. 10D). Although in these strong stilbene accumulators *PAL* transcripts were induced rapidly as well as *StSy* transcripts, the induction of *CHS* as key step for the flavonoid pathway remained transient and was shut off between 30 and 60 min after the UV-C pulse.

These patterns were then verified by quantitative real-time PCR in the same genotypes. For *RS* transcripts (Fig. 10B), no significant transcript accumulations can be detected under control conditions for none of the tested genotypes. However, already as early as 0.5 h, these transcripts had been clearly induced with the response of Hoe29, Ke53 and Ke83 being stronger than that of Augster Weiss, and this difference had magnified to almost a twofold difference at 6 h, when the induction in Ke53 is compared to Augster Weiss.

The basal levels for *CHS* transcripts (Fig. 10C) where higher in Augster Weiss and Ke83 compared to Hoe29 and Ke53. Irrespective of this initial difference, transcript levels increased transiently for 0.5 h in all genotypes, but this transient increase became significant only in case of Ke53. In all genotypes the transcript levels had dropped back at 6 h, for Hoe29, Ke53, and Ke83 even to a level lower than in the control. In case of Ke53, the transcripts almost vanished.

The pattern for *StSy* induction (Fig. 10D) resembled that for *RS* transcripts (Fig. 10B), but here, the induction was already quite pronounced at 0.5 h. Again, the *StSy* transcripts increased stronger and faster in Ke53 than in Augster Weiss. At 6 h, this difference had expanded to a level, where the expression of *StSy* in Ke53 was nearly twofold than that

observed in Augster Weiss.

3.1.7 Expression of *StSy*, *RS*, and *CHS* genes in response to downy mildew

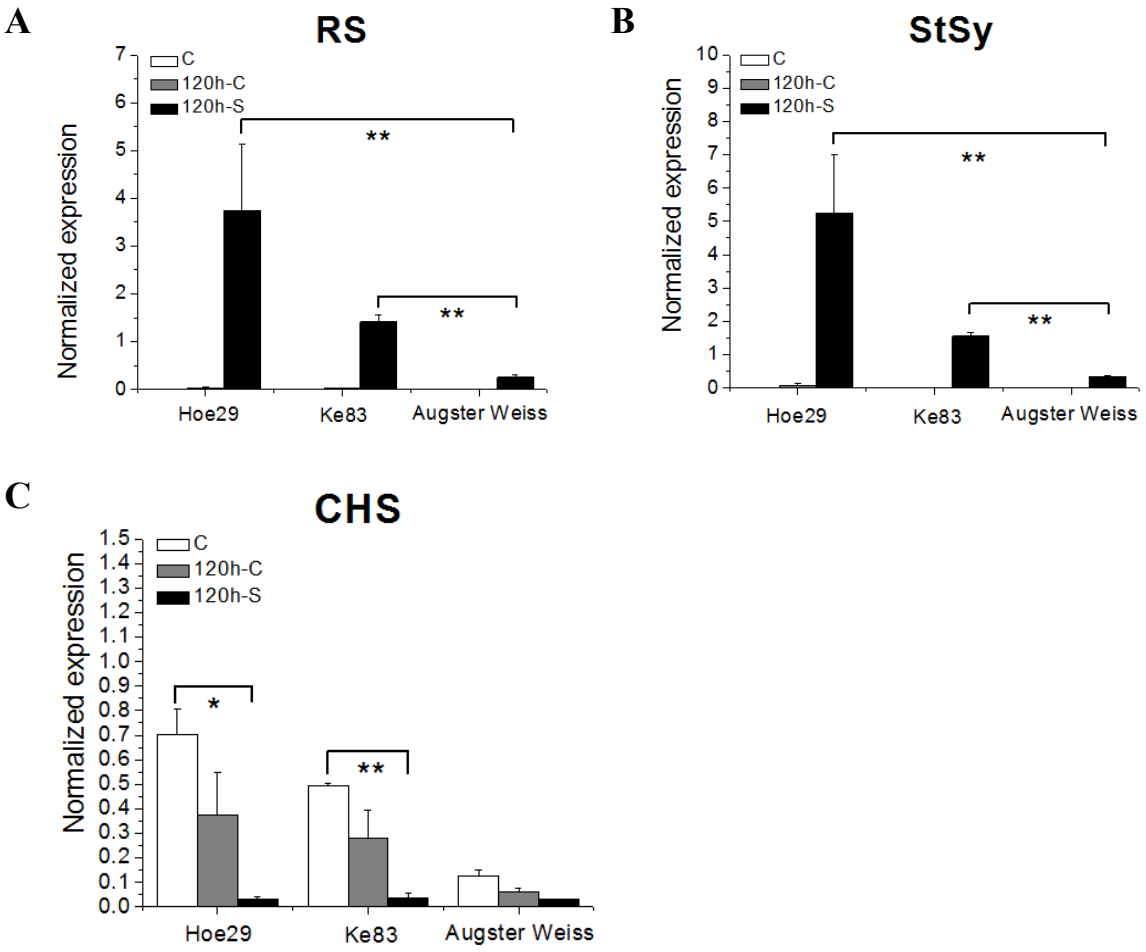


Fig.11 Response of key transcripts of the phenylpropanoid pathway to infection with downy mildew. (A-C) Quantification of transcripts of resveratrol synthase (*RS*), stilbene synthase (*StSy*) and chalcone synthase (*CHS*) by quantitative real-time PCR normalized to the expression of elongation factor *EF1-α*. * indicate differences that are statistically significant on the $P < 0.05$ level and ** indicate $P < 0.01$ level. Data represent mean values from three independent experimental series, error bars represent standard errors.

In the previous experiments, we had found genetic differences in the inducibility of stilbene inducibility that were accompanied by differences in the expression of stilbene synthase genes using UV-C as trigger. Since the motivation of this work was related to defence, we wanted to clarify, whether the observed induction by UV-C correlated with

an induction by downy mildew. For this purpose, the transcript levels of *StSy*, *RS*, and *CHS* were investigated by quantitative real-time PCR in three representative genotypes: Augster Weiss (a cultivated variety with weak stilbene induction in response to UV), and the two *V. sylvestris* genotypes Hoe29 and Ke83 that showed a strong stilbene response to UV.

For *RS* transcripts (Fig. 11A), for all three genotypes, no significant transcript accumulation was detected neither in the freshly excised leaf (C), nor in leaves that had been incubated for 5 days (120 h-C) without inoculation. However, 5 days after infection with downy mildew, the expression of *RS* in Hoe29 was strongly induced (by 131-fold compared to the control). This response was more than 14 times compared to Augster Weiss; also in Ke83, this induction was still nearly 6 times higher than in Augster Weiss.

The pattern of *StSy* (Fig. 11B) was similar to that for *RS* (Fig. 11A), here the expression of *StSy* in Hoe29 was 70-fold compared to the control and more than 15 times compared with Augster Weiss, and Ke83 was nearly 5 times than that observed in Augster Weiss.

In contrast, the abundance of *CHS* transcripts (Fig. 11C), irrespective of the initial difference, decreased compared to the C and 120 h-C for all genotypes. This was most pronounced in Hoe29 and in Ke83, where *CHS* transcripts were more abundant under control conditions. For Augster Weiss, the control levels were lower and thus the decrease was less prominent. Thus, the response *CHS* transcripts represented a mirror image of the situation observed for *RS* and *StSy*.

3.1.8 Susceptibility to downy mildew is inversely correlated with stilbene inducibility

For the tested representative genotypes the responses of *RS, StSy*, and *CHS* to inoculation with downy mildew (Fig. 11) correlated with the response of these transcripts to UV-C (Fig. 10). We therefore investigated a potential correlation between stilbene inducibility by UV-C and the susceptibility to infection by downy mildew over the population.

Plasmopara viticola infects through the stomata, and differences in stomatal density might therefore contribute to variations of infection success. We therefore screened our wild *Vitis vinifera* ssp. *sylvestris* Ketsch population for their stomatal densities.

Preliminary studies had shown that the relative incidence of stomata over the entire population of epidermal cells was a more reliable marker than absolute density (as stomata per area), because this relative value excludes variations caused by differences in cell expansion caused by environmental fluctuations (Table 4). In fact, the values for this relative stomatal density were found to be very stable over two vegetation periods, independent of lighting conditions, and dependent on the genotype.

Table 4 Stomatal density (as frequency of guard cell pairs per total number of epidermal cells) is independent of leaf expansion, leaf differentiation, and year. n indicates the number of leaves collected from different plants. From each leaf between 200-600 stomata were scored to determine stomatal density.

Genotype	**parameter**	**Stomatal density** [fraction of guard cells]
Ke110	Leaf expansion	
n = 8	Just emerged	0.092 ± 0.006
n = 4	During expansion	0.089 ± 0.005
n = 16	Fully expanded	0.092 ± 0.004
Ke83	Leaf differentiation	
n = 8	Small, green	0.060 ± 0.004
n = 4	Medium size, green	0.063 ± 0.003
n = 4	Large, green	0.062 ± 0.011
n = 16	Large, anthocyanin	0.065 ± 0.002
V. rupestris	Year	
n = 4	Year 1	0.099 ± 0.005
n = 16	Year 2	0.102 ± 0.003

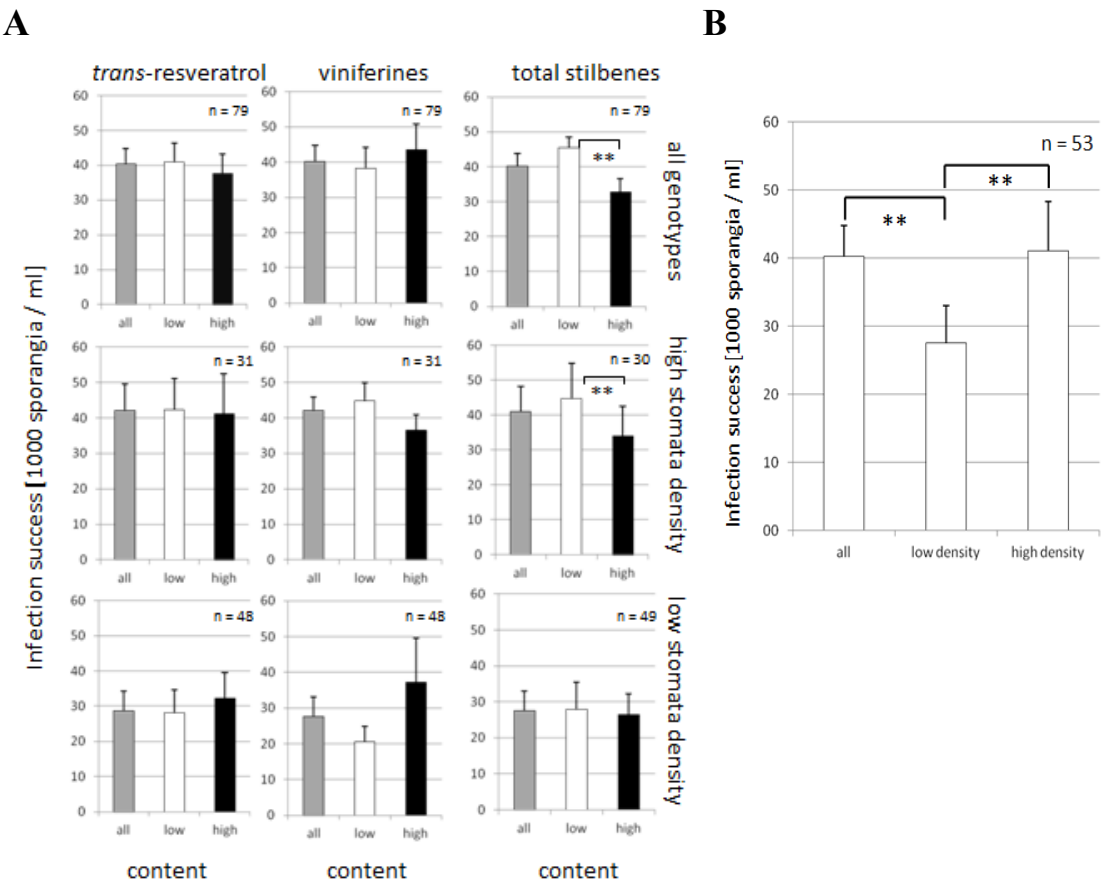


Fig.12 Correlation between UV-induced stilbene accumulation, susceptibility to downy mildew, and stomata density. (A) Mean susceptibility to downy mildew scored as concentration of sporangia formed by standardised inoculation in different subsets of the *V. sylvestris* population used in the current study. *all* pooled value over the entire population, *low* pooled value over those genotypes, where the abundance of the respective stilbene was lower than the average of the population, *high* pooled value over those genotypes where the abundance was higher than average. Upper row: all genotypes considered, middle row: only genotypes with high stomata density considered, lower row: only genotypes with low stomata density considered. (B) The comparison of average infection for all, low (below median of population), high (above the median of population) densities of stomata (ignoring any difference of stilbene level). ** indicate differences that are statistically significant on the $P < 0.01$ level. The data represent means and standard errors from three independent biological replicas obtained from at least two different years.

The entire population was now split into a (larger, n=59) subset where stilbene contents were lower than average and a (smaller, n=20) subset, where stilbene contents were

higher than average. As reference, we used the total abundance of resveratrol and viniferins at 24 h after induction. When now the concentration of sporangia was scored as readout for susceptibility and plotted over these stilbene subsets (Fig. 12A, upper row), there was no significant difference of infections if *trans*-resveratrol and viniferin were considered alone. Since resveratrol can be oxidized to viniferins also non-enzymatically during transport and storage of samples, we were analysing the correlation of infection with the sum of resveratrol and viniferins, because this value should be more robust against experimental fluctuations. Here, we found that the subset of high stilbene producers had significantly less infections compared to the subset of low stilbene producers. The significance is on the 99 % level.

Since genotypes with a low stomatal density are expected to suffer less penetration events, we also grouped the population into two subsets with respect to stomatal density – irrespective of stilbene inducibility (Fig. 12B), and found that there was a significantly reduced infection in the group with low stomatal density compared to the average of the entire population and to the high stomatal density group (significance on 99% level). We did not see a correlation between stomata density and stilbene inducibility, both traits seemed to be completely uncoupled.

Since the inverse correlation between stilbene levels and infection success was obscured by the fact that genotypes with low stomatal density are less infected even when they perform poorly with respect to stilbene induction, we tested the correlation between infection and stilbene levels separately for those genotypes with high stomatal density (Fig. 12A, middle row) and low stomatal density (Fig. 12A, lower row). Within this subset (Fig. 12A, middle row), the reduction of susceptibility in the high-stilbene producers was even more pronounced.

These data show that both, high stilbene inducibility, and low stomatal density confer a reduced susceptibility to downy mildew in the *V. sylvestris* population. For high stomatal density, the stilbene content is clearly limiting for infection success, whereas for low stomatal density, the infection success is mostly independent from stilbene content.

3.2 Differential regulation of *MYB14* in different genotypes

Höll *et al*. (2013) identified and functionally characterized two transcription factors (TFs) from grapevine, named *MYB14* and *MYB15*, which were found to be involved in the transcriptional regulation of stilbene biosynthesis. In our study, the specific differences were discovered by next-generation sequencing and confirmed by cloning for the *MYB14* promotor of representative genotypes, whereas *MYB15* did not reveal obvious changes. Therefore, we test the idea whether the strong inducibility of stilbene synthase transcripts in representative genotypes might result from elevated induction of *MYB14*.

3.2.1 Response of *MYB14* to UV-C and *P. viticola*

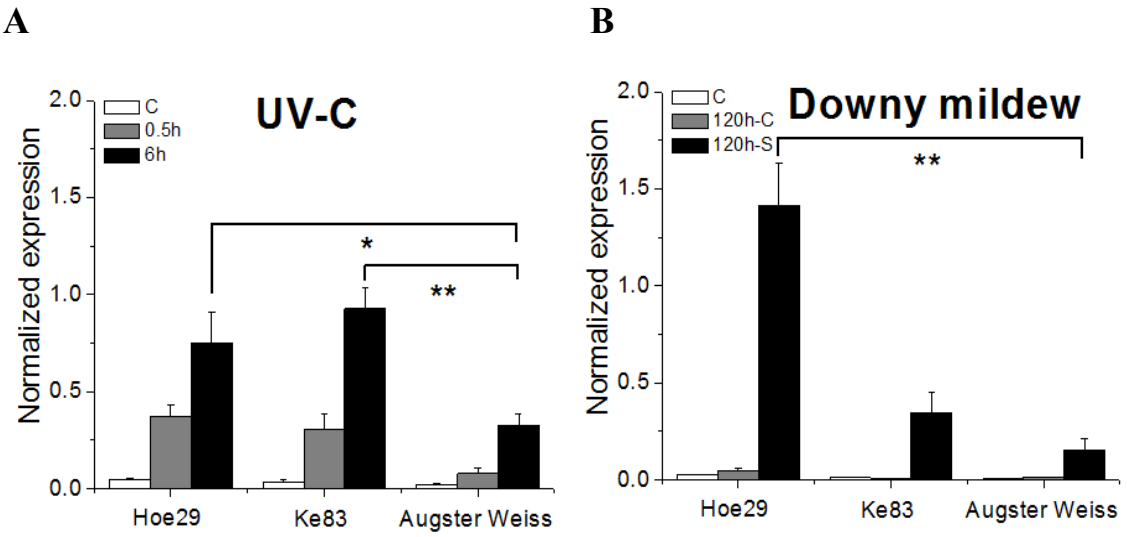


Fig.13 Expression of *MYB14* in response to UV-C and downy mildew infection. (A) UV-C irradiation for 10 min. (B) Downy mildew infection for 120 h. Quantification of transcripts by quantitative real-time PCR normalized to the expression of elongation factor *EF1-α*. * indicate differences that are statistically significant on the $P < 0.05$ level and ** indicate $P < 0.01$ level. Data represent mean values from three independent experimental series, error bars represent standard errors.

To illustrate that the transcription factor *MYB14* was potentially involved in the regulation of the stilbene branch in phenylpropanoid pathway, we followed the transcript level of *MYB14* in representative genotypes Hoe29, Ke83 and Augster Weiss in response to UV-C and *P. viticola* by quantitative real-time PCR.

As shown in Fig. 13A, no significant transcripts can be detected in controls for none of the tested genotypes. However, already as early as 0.5 h, these transcripts had been clearly induced with the response of Hoe29 and Ke83 being much stronger than the Augster Weiss. This difference had magnified at 6h, the induction of Hoe29 was (by 16-fold compared to control) more than 2 times compared to Augster Weiss; also in Ke83, this induction was still nearly 3 times compared to the Augster Weiss.

In Fig. 13B, 5 days after infection with downy mildew, the expression of *MYB14* in Hoe29 was strongly induced (by 30-fold compared to the control). This response was more than 9 times compared to Augster Weiss; also in Ke83, this induction was higher than Augster Weiss.

3.2.2 Oxidative burst was induced by UV-C

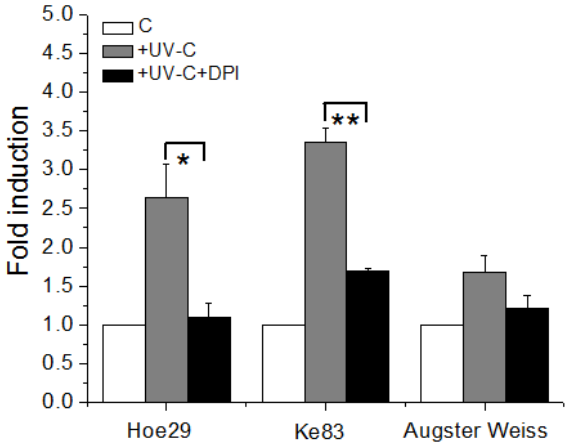


Fig.14 Effect of ROS on promoter activity of *MYB14* in response to UV-C. The columns show the fold induction levels of promoter activity in the presence of UV-C, UV-C with NADPH oxidase inhibitor (DPI) relative to the respective C (control) (promoter activity without any treatments). +UV-C: fold induction of promoter activity for 3 h after the UV-C irradiation 2 min; +UV-C+DPI: fold induction of promoter activity for 3 h after the addition of DPI (10 µM) and UV-C 2 min. Transient expression assay in *V. vinifera* cv Chardonnay suspension cell cultures after the particle bombardment. Specific promoter linked to a firefly luciferase gene was cobombarded into cells with a pART7 (empty vector) control. Each transfection assay contained the *Renilla* luciferase plasmid pRluc as an internal control. * indicate differences that are statistically significant on the $P < 0.05$ level and ** indicate $P < 0.01$ level. Mean values and standard errors from three independent experimental series.

The rapid generation of reactive oxygen species (ROS), termed oxidative burst, is an early inducible plant response during pathogen invasion or on the treatment with elicitors (Wojtaszek, 1997). To test whether the oxidative burst that was triggered by UV-C was necessary for the induction of *MYB14* promoters, the NADPH oxidase inhibitor DPI was used to quell the increase of ROS abundance following challenge with UV irradiation.

As shown in Fig. 14, the promoters' activities were induced in all genotypes after the UV irradiation, but they significantly increased in Hoe29 and Ke83 compared to Augster Weiss. After the application of DPI, the promoters' activities of *MYB14* were substantially suppressed in all genotypes and the inhibitions were much more pronounced in Hoe29 and Ke83, especially in Hoe29, nearly dropped to the control level.

3.2.3 Impact of JA on the activation of *MYB14* promoters

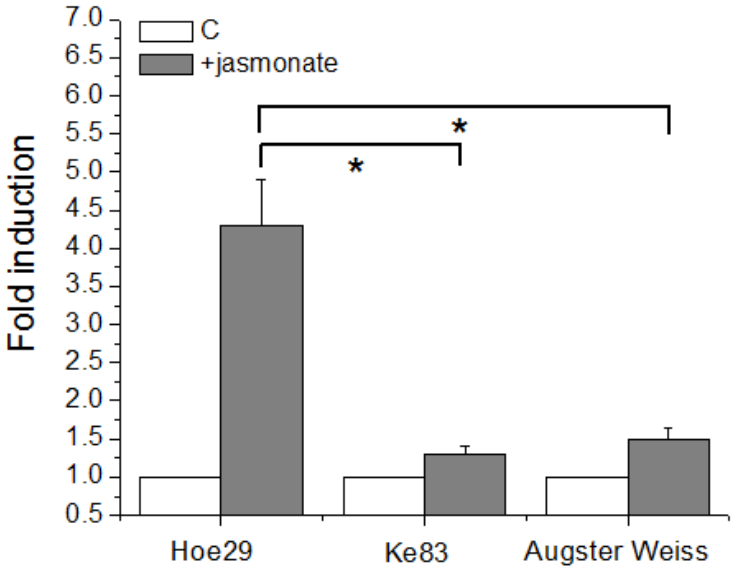


Fig.15 The activities of *MYB14* promoters in response to (±)-jasmonic acid (JA). The columns show the fold induction levels of promoter activities after the treatments of 50 µM (±)-jasmonic acid (JA) for 4 h. * indicate differences that are statistically significant on the $P < 0.05$ level. Mean values and standard errors from three independent experimental series.

It is well-known that jasmonic acid (JA) signalling is usually associated to herbivores, necrotrophic pathogens, as well as wounding (Bostock, 2005; Howe and Jander, 2008). In order to test whether these *MYB14* promoters respond to JA, cells were treated for 4 h

with 50 µM of JA. As a solvent control, cells were tested with a corresponding concentration of EtOH. As shown in Fig. 15, this induction was more pronounced in Hoe29 as compared with Ke83 and Augster Weiss. This result suggests that JA signalling is much more sensitive and necessary to mediate the activation of *MYB14* in Hoe29.

3.2.4 Impact of SA on the activation of *MYB14* promoters

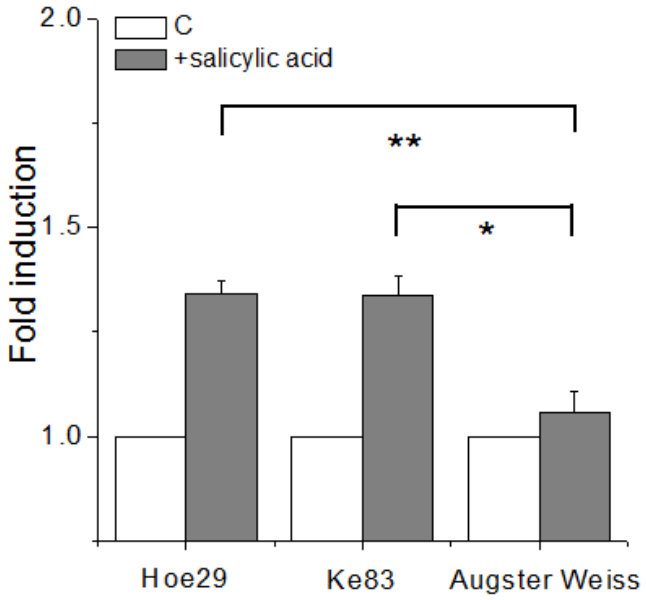


Fig.16 The activities of *MYB14* promoters in response to salicylic acid (SA). The columns show the fold induction levels of promoter activities after the treatments of 50 µM salicylic acid (SA) for 4 h. * indicate differences that are statistically significant on the $P < 0.05$ level and ** indicate $P < 0.01$ level. Mean values and standard errors from three independent experimental series.

As antagonist of JA signalling (Pieterse *et al*., 2012; Spoel *et al*., 2003), the SA pathway has acquired considerable interest. SA biosynthesis is triggered during both PTI and ETI (Bernoux *et al*., 2011; Mishina and Zeier, 2007), and often followed by activation of PR proteins, discussed with respect to systemic acquired resistance (Glazebrook, 2005; Grant and Lamb, 2006). In order to test whether these *MYB14* promoters respond to SA, cells were treated for 4 h with 50 µM of SA. As a solvent control, cells were tested with a corresponding concentration of EtOH. As shown in Fig. 15 and 16, the induction of SA observed in Hoe29 was weaker than JA; there was no much more difference of the induction observed in Ke83 compared to JA; the induction observed for SA was also

weaker than for JA in Augster Weiss. In Fig. 16, it was shown that the induction in Hoe29 and Ke83 was similar and both of them were more susceptible than Augster Weiss.

3.2.5 The activation of *MYB14* promoters in response to calcium ionophore

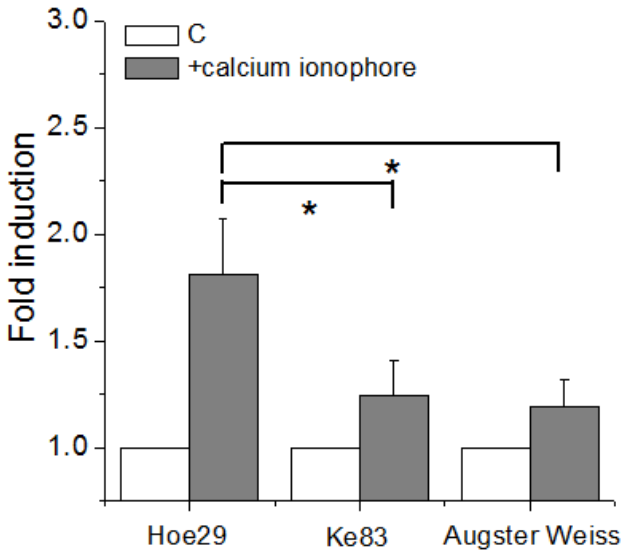


Fig.17 The activities of *MYB14* promoters in response to calcium ionophore. The columns show the fold induction levels of promoter activities after the treatments of 50 μM calcium ionophore for 4 h. * indicate differences that are statistically significant on the $P < 0.05$ level. Mean values and standard errors from three independent experimental series.

Ca^{2+} is an important second messenger of cells, which can regulate a variety of physiological reactions, such as plant growth, development and stress resistance (Bush, 1995). Calcium ionophore A23187 is a compound known as ionophore, which can form a complex with divalent cations. Because of its high selectivity for Ca^{2+}, it used as the important tool to study Ca^{2+} across the membrane (Pfeiffer *et al*., 1974). A23187 and Ca^{2+} constitute neutral compounds, which dissolve into the cell membrane and improve the permeability of cell membrane for Ca^{2+}, to form the high levels of intracellular Ca^{2+}. The increased Ca^{2+} as a second messenger can help plants improve the defence response in the process of interacting with pathogens. To test whether the Ca^{2+} was involved in the activation of *MYB14* promoters, we used the calcium ionophore to investigate the impact of calcium influx. As shown in Fig. 17, Hoe29, Ke83 and Augster Weiss had varying

degrees of increased promoter activity after the application of 50 µM calcium ionophore for 4 h. In particular, relative to Ke83 and Augster Weiss, the degree of Hoe29 increased significantly. This result suggests that the activation of *MYB14* in Hoe29 is more sensitive to the Ca^{2+} compared to Ke83 and Augster Weiss. As a solvent control, cells were tested with a corresponding amount of DMSO.

3.2.6 The activation of *MYB14* promoters in response to flg22

In order to investigate the earliest defence response, the potential differences of three genotypes, Hoe29, Ke83 and Augster Weiss, in response to flg22 were monitored. In Fig. 18, Chardonnay cell suspensions transiently expressing *MYB14* luciferase reporter constructs showed significant increases in the promoter activity of Hoe29 compared to Ke83 and Augster Weiss after incubation 4 h with flg22 (1 µM). In Hoe29, the activation increased to about 1.7 fold, whereas the induction was hardly detectable in Ke83 and Augster Weiss. This means the basal defence response is much more sensitive in Hoe29 compared to Ke83 and Augster Weiss.

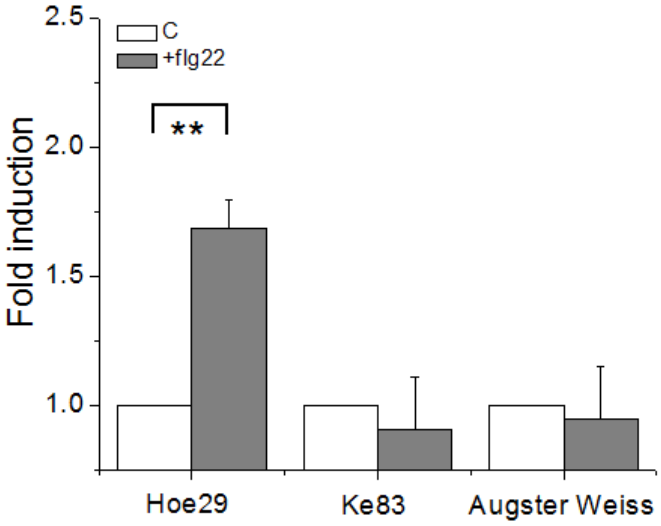


Fig.18 The activities of *MYB14* promoters in response to flg22. The columns show the fold induction levels of promoter activities after the treatments of 1 µM flg22 for 4 h. ** indicate differences that are statistically significant on the $P < 0.01$ level. Mean values and standard errors from three independent experimental series.

3.2.7 Different signalling pathways involved in flg22-triggered basal immunity for Hoe29

ROS is necessary

In order to test whether the ROS was involved in the process of flg22-triggered basal immunity in Hoe29, DPI was used to suppress the ROS production. As shown in Fig. 19A, flg22 could induce the increases of promoters' activities in Hoe29, but when the NADPH oxidase inhibitor DPI was added as well, the activation of *MYB14* promoters was significantly inhibited. In addition, there was no any significant change observed with the application of DPI separately. This result suggests that ROS is necessary for the flg22-triggered basal immunity in Hoe29.

Sensitive to Gd ions

Elicitors stimulate the secondary metabolism of plant cell response which needs molecular messengers, such as Ca^{2+} (Reddy, 2001), ROS (Neill *et al*., 2002b), transfer the stimulus information to the cells. The activity of a calcium influx channel is essential for the activation of early defence (Jabs *et al*., 1997) and should therefore be blocked by $GdCl_3$, an inhibitor of mechanosensitive calcium channels (Ding and Pickard, 1993). In our previous work, we had shown for cell cultures from *V. rupestris* and *V. vinifera* cv. 'Pinot Noir' that flg22-induced extracellular alkalinisation was more sensitive to Gd ions compared to Harpin-induced (Qiao *et al*., 2010; Chang and Nick, 2012).

In Fig. 17, we had found the activation of *MYB14* in Hoe29 was more sensitive to the Ca^{2+} compared to Ke83 and Augster Weiss. As shown in Fig. 18, the activation of *MYB14* promoters in response to flg22 was much more sensitive in Hoe29 and the ROS was to be tested participating in this process (Fig. 19A). Therefore, we would like to investigate whether the Ca^{2+} was also involved in the activation of early defence in response to flg22, we used the $GdCl_3$ to block the calcium influx channel. As shown in Fig. 19B, the promoter activity in response to flg22 was significantly inhibited by 20 μM $GdCl_3$. This finding suggests the Ca^{2+} is essential for flg22-induced activation of early defence in plant cells.

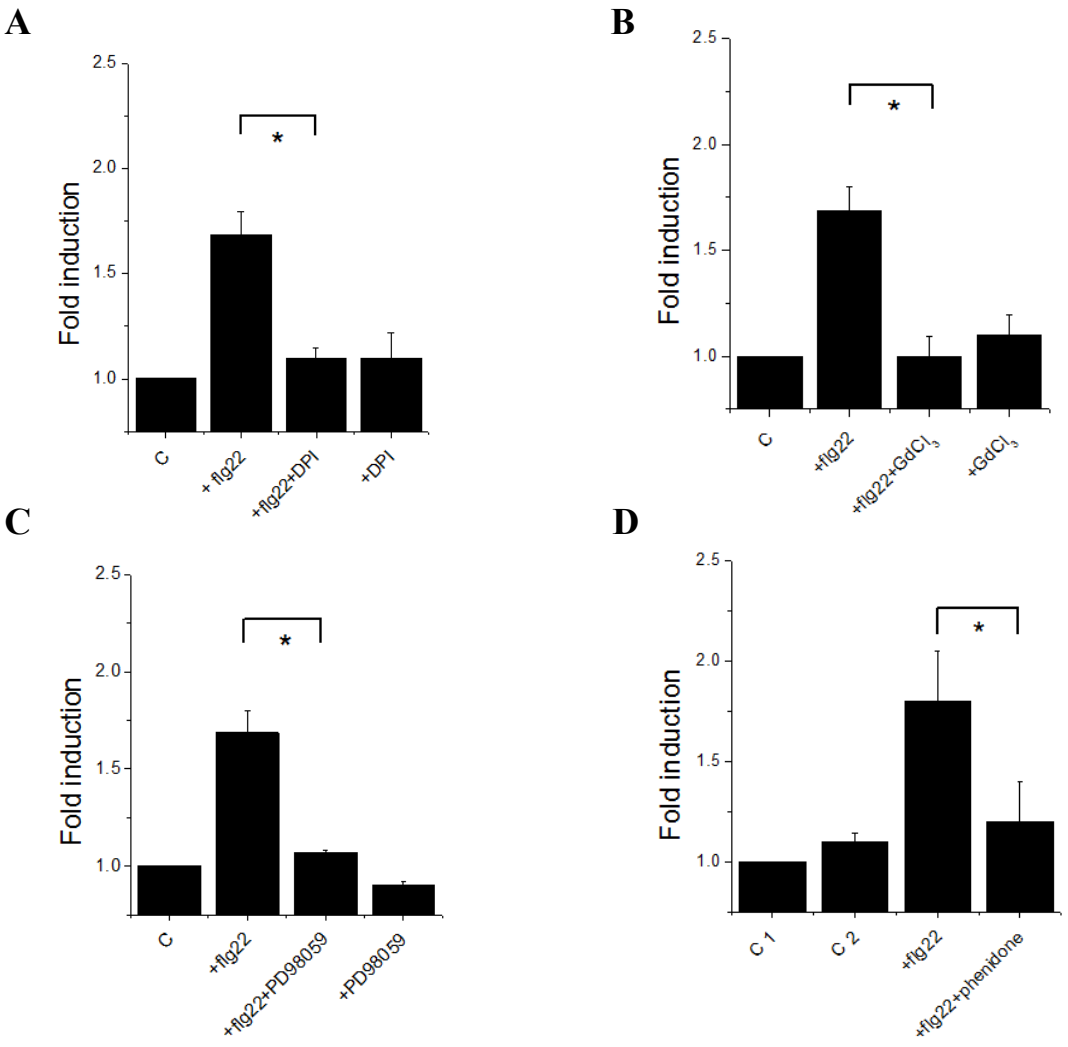


Fig.19 The effect of inhibitors on *MYB14* promoter activity (Hoe29) in response to flg22. (A) The columns show the fold induction levels of promoter activity after 4 h with the addition of 1 µM flg22, flg22 (1 µM) with NADPH oxidase inhibitor DPI (10 µM) or DPI (10 µM), separately. (B) The fold induction levels of promoter activity for 4 h after addition of 1 µM flg22, flg22 with 20 µM $GdCl_3$ or $GdCl_3$. (C) The fold induction levels of promoter activity for 4 h after addition of 1 µM flg22, flg22 with 100 µM MAPK cascades inhibitor PD98059 or PD98059. (D) The columns show the fold induction levels of promoter activity pre-treated with phenidone 2 mM containing 0.1 % Tween® 20 for 30 min in response to 1 µM flg22 for 4 h. C1 (Control 1) was pre-treated with 0.1 % Tween® 20 for 30 min before 0 µM flg22 for 4 h; C2 (Control 2) was pre-treated with 2 mM phenidone containing 0.1 % Tween® 20 for 30 min before 0 µM flg22 for 4 h. * indicate differences that are statistically significant on the $P < 0.05$ level. Mean values and standard errors from three independent experimental series.

MAPKKS activity is necessary

The Mitogen-activated protein kinase (MAPK) cascades represent one of the major signalling systems of eukaryotic cells. The MAPK cascades were implied in the activation of defence gene expression in several studies (Zhang and Klessig, 2001; Pitzschke and Hirt, 2006) and also shown associated with the induction of plant defence responses (Zhang and Klessig, 2001; Jonak *et al*., 2002). To test whether the MAPK signalling was involved in the early defence, PD98059, a specific inhibitor of the mitogen-activated protein kinase (MAPK) cascades, was used to probe for a potential feedback of MAPK signalling. As shown in Fig. 19C, for flg22 induced activation of *MYB14* promoters, an obvious decrease was observed after the addition of 100 μM MAPK cascades inhibitor PD98059. Therefore MAPK signalling is necessary for flg22-triggered basal immunity and activation of *MYB14* promoters in Hoe29.

Phenidone can inhibit the induction

JA signalling was much more susceptible and necessary to mediate the activation of *MYB14* in Hoe29 as shown in Fig. 15. In order to investigate further whether the induction triggered by flg22 also required JA, the synthesis of JA was blocked by phenidone, an inhibitor of LOXs that trigger early steps in the octadecanoid pathway. The cells were pre-treated with 2 mM phenidone for 30 min before adding 1 μM flg22 for 4 h. As controls, cells were pre-treated with 0.1 % Tween® 20 or 2 mM phenidone containing 0.1 % Tween® 20 for 30 min before 0 μM flg22 for 4 h (Fig. 19D). Phenidone inhibited the flg22-triggered induction of *MYB14* promoters in Hoe29 very efficiently. This result suggests that JA signalling is necessary to mediate the activation of *MYB14* in response to flg22.

4. Discussion

Stilbenes, as important phytoalexins, are a central factor for basal immunity of grapevine. In this study, we probed for potential genetic variation in *V. sylvestris*, the ancestor of cultivated grapevine with respect to their stilbene biosynthetic capacities, for potential use for resistance breeding. We show genotypic differences in abundance and profiles of the stilbenes induced in response to a UV-C pulse. Two clusters of genotypes emerged: one cluster with quick and strong accumulation of stilbenes, almost exclusively in form of the non-glycosylated resveratrol and viniferins. The second cluster accumulated less stilbenes and relatively high proportion of piceatannol and the glycosylated piceid. For all 86 genotypes we observed a time-dependence of the stilbene pattern: piceid, resveratrol and piceatannol accumulated earlier, whereas the viniferins were found later consistent with a mode of action, where resveratrol acts as precursor for the viniferins. We further observed that the genotypic differences in stilbene accumulation were preceded by differential accumulation of the transcripts for *PAL*, *StSy*, *RS* and *CHS*. Taken together, these observations provide evidence for stilbene "chemovars" in *V. sylvestris* (and possibly also in the few vinifera cultivars tested in this study) that differ with respect to the induction of bioactive viniferins correlated with a difference in the inducibility of stilbene synthase.

Furthermore, we asked, whether the induction of stilbene synthase transcripts might result from elevated induction of *MYB14* and *MYB15*. Specific differences were discovered in the *MYB14* promotor of Hoe29 by next-generation sequencing, whereas the *MYB15* did not reveal obvious changes. Subsequent cloning confirmed that both *V. sylvestris* genotypes harbour certain common domains (in addition to domains that differ between Hoe29 and Ke83), which were absent from the promotor of the cultivated variety Augster Weiss (that is a weak stilbene producer). We can show that the differences in stilbene synthase inducibility correlate with differences in the induction of *MYB14* transcripts. To understand the functional relevance of these specific promotors and to map the upstream signalling, we employed a promotor-reporter assay (Höll *et al.*, 2013). We show that both *V. sylvestris* promotors (but not the promotor from the weak stilbene producer Augster Weiss) are induced by UV light in cell culture, and this induction requires the activity of a

NADPH oxidase. However, only the *MYB14* promotor from Hoe29, was induced by flg22 and jasmonic acid (again dependent on the activity of a NADPH oxidase) indicating that here, *MYB14* was associated with PTI signalling. In contrast, the *MYB14* promotor from Ke83, although inducible by UV light, was not induced in the context of PTI. This is consistent with our findings, where *StSy* transcripts were strongly induced by UV light in both genotypes, whereas for infection by *P. viticola* induction was observed only in Hoe29, but not in Ke83.

4.1 On what level is stilbene accumulation controlled?

In our study, the genotypes from the "blue" (high-stilbene type) cluster (Fig. 6A), such as Pinot Noir, Pinot Blanc, Ke15, Ke20, Ke22, Ke39, Ke53, Ke83, Ke84, Ke95, Ke96, Ke99, Ke103, Hoe17 and Hoe29, accumulate high levels of stilbenes in response to a UV-C pulse (Fig. 4B and Fig. 4C, the dots on the top of the boxplot at 24 h), but all show only very low basal levels of stilbenes in control conditions. This means that these genotypes produce their strong induction of stilbenes completely through *de novo* synthesis.

Since stilbenes are derived from the phenylpropanoid pathway (Fig. 2), the general activation of this pathway was monitored by probing for *PAL*. During evolution, the stilbene branch of the pathway has branched from flavonoid biosynthesis by duplication of the gene encoding *CHS* followed by mutation in the active centre giving rise to *StSy*/*RS* (Tropf *et al*, 1994). The difference between these enzymes triggering the competing branches of stilbene versus flavonoid biosynthesis are very similar, with only one amino-acid difference in the active centre, and the substrate of *StSy*/*RS* is also used by *CHS*, such that both pathways compete for the same precursor. As shown for representative genotypes in Fig. 10A, in all strong stilbene accumulators tested, the induction of *PAL* transcripts was accompanied by an almost simultaneous induction of *StSy* transcripts, whereas *RS* transcripts followed 1-2 h later. In contrast, this response was delayed in Augster Weiss and less pronounced as compared to the strong stilbene accumulators. This indicates that the genotypic differences in the accumulation of stilbenes (Fig. 10) are correlated with the induction of *PAL* transcription as key regulator of the entire phenylpropanoid pathway. Interestingly, in these strong stilbene

accumulators, the *CHS* as key enzyme for the flavonoid pathway, although initially slightly induced by UV-C as well, was subsequently down regulated. This indicates that the phenylpropanoid pathway is, upon activation by UV-C, channelled towards the synthesis of stilbenes, whereas the flavonoid pathway, although initially activated, is rapidly shut down. This might be linked with differential recruitment of *MYB* transcription factors to the *CHS* and *StSy* promotors (Höll *et al.* 2013).

Although there is a clear correlation between differential activation of *StSy* transcription and the accumulation of stilbenes, it is also clear that the differential induction of *StSy* transcripts (not exceeding a factor of 2-3) can not account for the much larger differences in the induction of stilbenes (up to a factor of 20). This indicates that transcriptional regulation must be complemented by (still unknown) posttranscriptional mechanisms consistent with findings from elicited grapevine cell lines, where activation of basal immunity by the PAMP flg22 produced a strong accumulation of *StSy* transcripts that were not followed by accumulation of stilbenes (Chang and Nick, 2012). In contrast, the bacterial elicitor Harpin, triggering a cell-death related version of immunity, induced *StSy* transcripts to a similar level, but in addition caused a strong accumulation of stilbenes. An important role of posttranscriptional regulation is also suggested by the fact that a cell culture of Pinot Noir, a genotype belonging to the high-stilbene type cluster, preferentially produce the glycosylated piceid upon induction of defence (Chang *et al*. 2011) indicating that epigenetic mechanisms modulate the phenotype.

4.2 Can resistance to downy mildew be predicted by UV-C inducibility of stilbenes?

To analyse stilbene inducibility on a comparative scale, a pulse of UV-C was used as reliable and standardised input. However, the motivation for this study was to explore the potential of *V. sylvestris* as genetic resource for resistance breeding. This required to probe for potential correlations between UV-C response and the response to a pathogen, such as downy mildew. This correlation is supported by two lines of evidence: 1. The patterns for the induction of stilbene synthesis transcripts (*RS*, *StSy*) along with the

competing flavonoid pathway (probed by *CHS*) are highly congruent, irrespective, whether UV-C or inoculation with *P. viticola* are used as trigger. 2. Those genotypes that produce high levels of stilbenes in response to UV-C are also found to be significantly less susceptible to infection with downy mildew as compared to those genotypes with low UV-inducibility of stilbenes. This correlation becomes even tighter, when genotypes with high stomatal density are considered. Thus, the inducibility of stilbene synthesis by a UV-C pulse can be used as predictor for (partial) resistance to infection with downy mildew.

4.3 The earliest cellular response of basal immunity

Plants respond to various biotic and abiotic stresses by different, often very specific responses. This requires that the signalling triggered by a specific stress has to be specific and distinct. A straightforward mechanism to ensure this would be the use of separate, distinct signalling molecules that convey the signals triggered by the different stress factors. However, it seems that specificity is brought about by only a limited number of molecular players that even overlap in their activity, such as Ca^{2+}, reactive oxygen species, and jasmonates. When such a small number of components can cause a specific response, there must be other aspects of signalling (beyond the molecular nature of the signal) defining specificity. As attractive possibility, the integration of spatiotemporal activity patterns of these molecules (so called “stress signatures”) might lead to differential output. The best known example for such a signature model is calcium, where, by means of aequorin-reporter plants, stress-quality specific patterns of calcium spiking were discovered (Knight *et al*., 1991; McAinsh and Hetherington, 1998). By rhythmic incubation with calcium-free versus calcium-rich buffers, it could be shown later for stomatal aperture as example that not calcium as a molecule, but the temporal pattern of its abundance confer the signal (Pei *et al*., 2000). Similar signature models have been proposed for other stress signals, such as reactive oxygen species (Miller *et al*., 2010), jasmonate (Kazan and Manners, 2009), or microtubules (Nick 2013).

Plant immunity represents a striking example for the impact of signatures: Although most of the initial cellular events are shared, the output can be radically different: a basal layer

of immunity effective against a broad range of pathogens can be complemented by a pathogen-specific layer of immunity that often culminates in programmed cell death as efficient strategy against biotrophic pathogens. This bifurcation also holds true for grapevine cell cultures, where these two forms of immunity can be evoked using different elicitors, and where most of the early signals are identical, but just shifted in their temporal order (Chang and Nick 2012). Thus, efficient induction of immunity requires correct timing of early signalling.

In our work, we had identified some genotypes in *V. sylvestris*, the wild ancestor of cultivated grapevine, which can accumulate antimicrobial stilbenes, the major phytoalexin of grapevine, very efficiently. This trait was correlated with a rapid accumulation of stilbene-synthase transcripts. For those genotypes, where this was tested, both UV light as well as inoculation with downy mildew were effective as inducing factors indicating that early signals shared between these two stress factors must be involved.

We therefore asked, whether the induction of stilbene synthase transcripts might result from elevated induction of *MYB14*, a transcription factor, activating the stilbene-synthase promotor (Höll *et al.*, 2013). Focussing on the *V. sylvestris* genotype Hoe29, which in our study had turned out to accumulate resveratrol and viniferins, efficiently and rapidly, next-generation sequencing revealed significant deviation of the *MYB14* promotor sequence from the *vinifera* reference genome, whereas the *MYB15* promotor from the same genotype analysed for comparison did not reveal obvious changes. Subsequent cloning confirmed that both tested *V. sylvestris* genotypes (Hoe29 and Ke83, a second genotype, which had been identified as promising in our work) harbour certain common domains (in addition to domains that differ between Hoe29 and Ke83), which were absent from the promotor of the cultivated variety Augster Weiss (as weak stilbene producer). We can show that the differences in stilbene synthase inducibility correlate with differences in the induction of *MYB14* transcripts. To understand the functional relevance of these specific promotors and to map the upstream signalling, we employed a promotor-reporter assay (Höll *et al.*, 2013). We show that both *V. sylvestris* promotors

(but not the promotor from the weak stilbene producer Augster Weiss) are induced by UV light in cell culture, and that this induction requires the activity of a NADPH oxidase. However, only the *MYB14* promotor from Hoe29 was also induced by jasmonic acid and flg22 (again dependent on the activity of a NADPH oxidase) indicating that this allele of *MYB14* was additionally target of the signalling driving basal immunity (PTI). In contrast, the *MYB14* promotor from Ke83, although inducible by UV light, was not induced in the context of PTI. This is consistent with our findings, where *StSy* transcripts were strongly induced by UV light in both genotypes, whereas for infection by *P. viticola* induction was observed only in Hoe29, but not in Ke83. We will, in the following, present a signature model of immunity signalling that can explain most, if not all of our observations (Fig. 20):

The earliest known cellular response of basal immunity is the activation of a rapid influx of Ca^{2+} and H^{+} (Nürnberger, 1999). In case of the PAMP flg22, perception is brought about by the receptor FLS2 (Boller and Felix, 2009; Robatzek and Wirthmueller, 2013), for which also a grapevine homologue has been described (Di Gaspero and Cipriani, 2003). An immediate target of activated FLS2 signalling is cyclic-nucleotide gated calcium influx channels (Ma and Berkowitz, 2011). Binding of flg22 to the receptor will therefore result within few minutes in a substantial increase of cytosolic calcium (Fig. 20,①).

Calcium as important second messenger plays an important role as activator of various signal chains (Harper and Harmon, 2005). One of these secondary signalling events is the stimulation of the membrane-bound NADPH oxidase RboH through specific calcium-dependent protein kinases (Kobayashi *et al.*, 2007), leading to an apoplastic oxidative burst generating superoxide anions (Fig. 20,②). Superoxide anions represent the second central input for plant stress signalling (Marino *et al.*, 2012), and can also be formed in response to UV light (Hideg *et al.*, 2012). The observation that both *V. sylvestris* alleles of the *MYB14* promotor show elevated activation by UV that can be blocked by DPI (Fig. 14) suggests that this induction is triggered by RboH. In contrast, only the Hoe29 allele, but not the Ke83 allele, was activated by flg22 (Fig. 18). This

activation was also dependent on the activity of RboH, because it can also be blocked by DPI (Fig. 19A), suggesting that RboH acts downstream of flg22 or complements signalling triggered by flg22. Since activation of RboH by UV light can activate also the Ke83 allele of the *MYB14* promotor, it would be expected, at first sight, that flg22 can also activate this promotor allele, because the upstream signalling is provided by the identical recipient (suspension cells of 'Chardonnay') in the promotor-reporter system. This means that the differential activation of the two *V. sylvestris* promotors must be caused by differential activation with other branches of signalling that are independent of RboH. From our model we would predict that these RboH signalling events are activated by calcium influx directly. To test this prediction, we used a calcium ionophore and observed that this significantly activated the Hoe29 allele, whereas the Ke83 and Augster Weiss alleles did not produce significant activation (Fig. 17). When the flg22-triggered influx of calcium is blocked by Gd^{3+} ions, a specific inhibitor of mechanosensitive calcium channels (Ding and Pickard, 1993), this will also block the activation of the Hoe29 allele of the *MYB14* promotor (Fig. 19B). Calcium influx is therefore necessary and sufficient for this activation.

In addition to activation of RboH through a calcium-dependent protein kinase, cytosolic calcium triggers two additional signalling chains: One of the targets is a MAPK cascade that conveys the signal from the membrane to the nucleus. This MAPK cascade pathway is highly conserved in eukaryotes, and is composed of three hierarchical layers, whereby MAPK kinase kinases phosphorylate MAPK kinases, which in turn activate MAP kinases that will then activate different downstream targets. This pathway is central for basal immunity (Nürnberger *et al*., 2004) and also mediates the activation of grapevine stilbene synthase in response to flg22, as concluded from experiments with the specific MAPK inhibitor PD98059 (Chang and Nick, 2012). We therefore tested, whether PD98059 can block the induction of the Hoe29 allele of *MYB14* by flg22. This was, what we observed (Fig. 19C), showing that the MAPK cascade is necessary.

There is an alternative calcium-dependent branch of defence signalling, though (Fig. 20,④): Calcium reaching the plastid will activate there specific lipoxygenases

(Wasternack and Hause, 2013) as first committed step of the oxylipin pathway generating jasmonates. In *Arabidopsis*, mutants affected in vacuolar calcium channels (Bonaventure *et al.*, 2007) fail to activate AtLOX2, the lipoxygenase, which is the central trigger for the oxylipin pathway. The molecular mechanism is not clear, but might be linked with the binding of lipoxygenase to the membrane due to a conserved calcium binding loop interspersed between two β-sheets (Tatulian *et al.*, 1998). This will alter the specificity of lipoxygenase–whereas the free enzyme converts linoleic acid to conjugated dienes, upon binding to the membrane, it preferentially forms a conjugated ketodiene. In consequence, within a few minutes, cis-(+)-12-oxophytodienoic acid (OPDA) is exported from the plastid and converted to jasmonic acid (JA) and its potent conjugate JA-Ile.

We therefore tested, whether exogenous jasmonic acid could activate *MYB14* in the absence of flg22. This is in fact, what we can observe (Fig. 15), whereby this activation only works with the Hoe29 allele, whereas the alleles from Ke83, and Augster Weiss are not responsive to jasmonic acid. To test, whether induction of (endogenous) jasmonic acid is necessary for this activation, we treated the cells with phenidone, an inhibitor of jasmonate synthesis targeted to lipoxygenases (Ismail *et al.*, 2012), and we found that phenidone can block the flg-22 induced activation of *MYB14*. Thus, jasmonate is necessary and sufficient to convey the activation of flg22 to the Hoe29 allele.

This points to a scenario, where flg22 activates the MAPK cascade as well as jasmonate signalling that converge on a target on the *V. sylvestris MYB14* promotor that is present in Hoe29, but not in Ke83. Nevertheless, RboH seems to be necessary as well and this effect of RboH seems to be different from that in the UV-activation of *MYB14* (which was similar in both *V. sylvestris* alleles of this promotor). This apparent discrepancy can be resolved, when RboH dependent signalling converges with jasmonate synthesis. This point of convergence might be again the lipoxygenase that not only is activated by calcium, but requires hydrogen peroxide (Fig. 20,③). Hydrogen peroxide is generated in the peroxisomes from superoxide, and is then further converted to water by catalase **(**Fig. 20,③). It has been known for a long time that the activity of catalase can be inhibited by the important stress factor salicylic acid, leading to elevated levels of hydrogen peroxide

(Durner and Klessig, 1996).

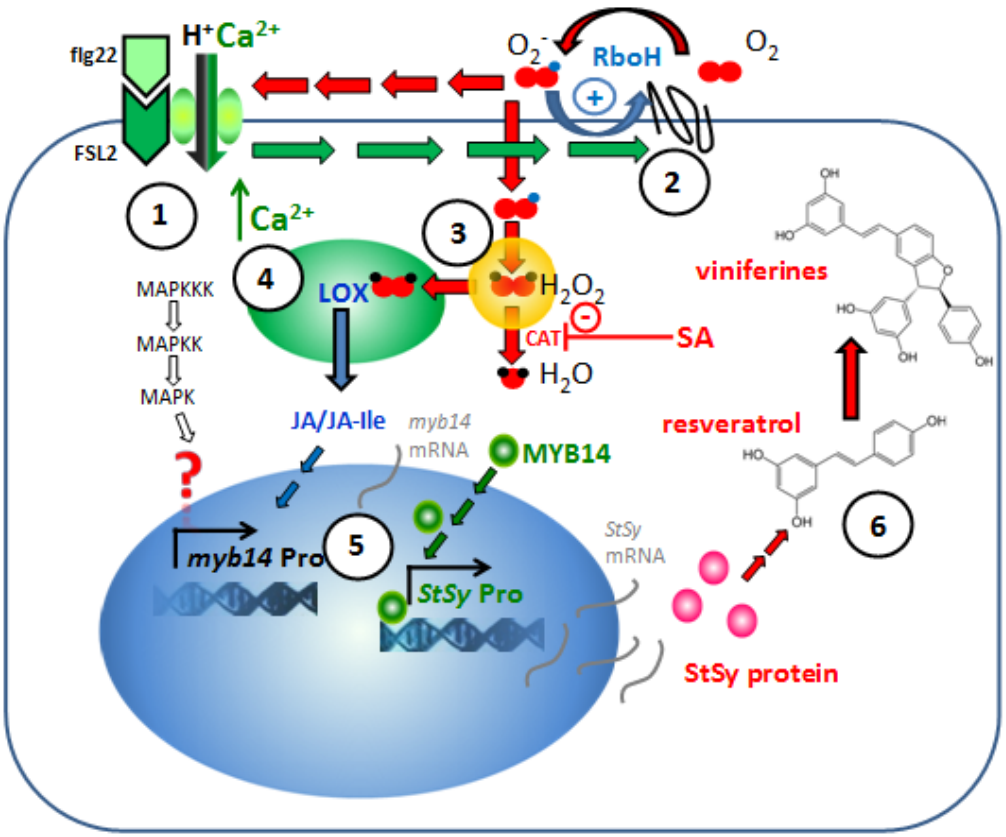


Fig.20 The signature model of immunity signalling. Details are explained in the discussion. ① Binding of flg22 to the receptor will therefore result within few minutes in a substantial increase of cytosolic calcium. ② The stimulation of the membrane-bound NADPH oxidase RboH through specific calcium-dependent protein kinases, leading to an apoplastic oxidative burst generating superoxide anions. ③ Hydrogen peroxide is generated in the peroxisomes from superoxide. ④ Calcium reaching the plastid will activate there specific lipoxygenases as first committed step of the oxylipin pathway generating jasmonates. ⑤ *MYB14* transcription factor. ⑥ Stilbenes.

Our model would therefore predict that salicylic acid, by blocking the reduction of hydrogen peroxide, should promote the activation of lipoxygenase, and therefore also the activation of the Hoe29 allele of *MYB14* by flg22 should depend on RboH. We have tested this prediction experimentally and found that DPI can block the activation (Fig. 19A), consistent with the prediction. We have further found that salicylic acid can activate the Hoe29 allele of *MYB14* (Fig. 16). However, the activation was observed for both *V.*

sylvestris alleles pointing to additional targets of salicylic acids (different from jasmonate). But, since this activation was also very weak (although significant), the impact of salicylic acid alone (i.e. without synergy with flg22) seems to be fairly marginal.

4.4 Outlook

In grapevine, stilbenes are central to defence response, especially resveratrol effectively prevents pathogen attack (Jeandet *et al*., 2002; Adrian *et al*., 1997). Resveratrol is complemented by other metabolic compounds, which harbour efficient antimicrobial activities and are also induced in grapevine as a result of infection or stress (Langcake, 1981; Pezet *et al*., 2004). Among all stilbenes, oxidized resveratrol oligomers, so called viniferins are even more toxic than resveratrol itself and have been shown to inhibit zoospore mobility of *Plasmopara viticola*. In contrast, piceid – the glycosylated form of resveratrol – shows no or little toxicity and no antimicrobial activity (Celimene *et al*., 2001; Pezet *et al*., 2004). Although stilbenes were induced in all 86 genotypes in response to the UV-C pulse, the genotypes from the blue cluster (Fig. 6A) differed from those of the green cluster not only in accumulating higher levels of stilbenes, but in addition produced the non-glycosylated bioactive stilbenes resveratrol and viniferin. We are currently exploring the performance of the *V. sylvestris* genotypes after inoculation with different grapevine pathogens such as *Plasmopara viticola*, *Erysiphe necator* or *Guignardia bidwellii* and find statistically significant correlations between stilbene accumulation and suppression of disease symptoms.

The fact that it is possible to induce stilbene accumulation via an abiotic stress factor (a pulse of UV light) opens the interesting possibility that immunity might be stimulated by appropriate pretreatments with abiotic factors. The induction of tolerance to a certain type of stress by a controlled induction of a different stress pathway is termed as stress priming and has attracted considerable attention in the context of improving agronomical performance under adverse conditions (Beckers and Conrath, 2007). Our study demonstrates that genetic factors enabling strong stilbene inducibility are still present in *V. sylvestris*, and might be reintroduced into cultivated grapes. Since viticulture is not targeted to provide staple food, but a high-quality, high-priced product, quality has clear

priority over bulk production. The expected (slight, because inducible) costs for growth and yield expected upon reinstallment of stilbene inducibility would be more than compensated by the reduced costs for chemical plant protection, reduced loss by pathogens, and improved sustainability. Since the “blue” (high-stilbene type) genotypes seem to cluster to specific branches of the phylogenetic tree constructed for the European Wild Grape, we also want to explore the possibility to use the ancestor of cultivated grapevine as genetic resource for marker-assisted breeding for improved basal immunity.

Based on the previous findings, we propose a mechanism in the next step to explain the observed phenotype of a specific *sylvestris* genotypes, Hoe29, and draws a link between specific regions in the promotor of the transcription factor *MYB14*, elevated inducibility of this promotor by the signalling activated during basal immunity, and the observed strong accumulation of resveratrol and viniferins correlated with the improved tolerance of these genotypes against downy mildew. Although we can reproduce the response patterns observed in the plant in the promotor-reporter system, for instance the differential activation of stilbene synthase transcription in the Hoe29 versus the Ke83 genotypes, the general activation of the promotor is lower than the observed accumulation of transcripts *in planta*. This indicates that differentiated grapevine cells harbour enhancing factors that are not present in non-differentiated suspension cells. A similar phenomenon with similar ratios is observed for the stilbene synthase, when the induction of transcripts in planta are compared to the inductions observed in the promotor-reporter system (Höll *et al.*, 2013). Whether these factors are of epigenetic nature or simply additional signalling factors remains to be elucidated. Also, the role of the *MYB15* factor should be addressed as well as *MYB14*-independent direct signalling to the stilbene synthase promotor. These aspects are currently analysed and are expected to complement and refine the proposed working model. Irrespective of the exact signalling networks, the Hoe29 allele of *MYB14* can be analysed further to define targets for molecular breeding of grapevine varieties with elevated basal immunity due to inhanced *MYB14* inducibility.

Currently, we have got the crosses of Hoe29 x Augster Weiss and Ke83 x Augster Weiss, as well as their genomic DNA from Dr. Rudolf Eibach in Julius Kühn Institute (JKI),

Germany. In order to evaluate the genetic inheritance in breeding plants, we designed specific primers (Appendix 5.8) to investigate the offspring making use of the promoter sequence differences of *MYB14* present in Hoe29, Ke83 and Augster Weiss (an ancient cultivar, which is male sterile and therefore well suited for breeding). At the same time, we will investigate the stilbene inducibility in breeding offsprings after the UV irradiation and infection by *P. viticola*. These reulsts will show us more information about the overall correlation between the biochemical and genetic aspects. Those individuals which inherited *MYB14* promoter from Hoe29 might be good candidates to further improve the success of sustainable viticulture in the future.

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5. Appendix

5.1 The extraction of genomic DNA

1. Prepare the 2% CTAB (W/V) extraction buffer including the 1.4 mol/L NaCl, 150 mmol/L Tris and 30 mmol/L EDTA, setting the pH at 8 with 1 mol/L HCl.
2. Take 2 ml eppendorf tubes and put 5 mm beads in.
3. Give 50- 60 mg of fresh leaves or 10- 20 mg dried samples in the tube and freeze them immediately in the liquid nitrogen.
4. Add 8 µl/ml beta-mercaptoethanol and 40 mg/ml PVP (Polyvinylpyrrolidone) (Sigma MW 10,000) to the CTAB-buffer, melting at 60 °C.
5. Shred the frozen samples with tissuelyzer (15 sec, 20 HZ, two times).
6. Add 900 µl CTAB mixed buffer (with beta-mercaptoethanol and PVP) to each sample (vortex and mix uniformity) and incubate 15 min at 60 °C; during the incubation, mixing the sample every 5 min by hands.
7. Add 100 µl Proteinase K (1 mg/ml) (Merk, Germany) in the sample and incubate 20 min at 58 °C; and then cool samples down at room temperature.
8. Add 750 µl of chloroform: IAA (isoamylalcohol) (24:1); mix gently until it gets emulsion and then centrifuge 10 min at 14 000 g.
9. Take 750 µl of the supernatant into 1.5 ml new eppendorf tubes; add 7 µl RNase A (10 mg/ml) to each sample and incubate 30 min at 37 °C.
10. Add isopropanol into the sample which volume is equal to 60 % of the sample, and mix it careful without shaking; incubate it 30 min at -20 °C (mix the samples while incubating); the sample also can be hold at the -20 °C over night; centrifuge 10 min at 14 000 g.
11. Take away the supernatant/overlap (CTAB-waste) completely and wash the pellet 2 times with 500 µl of 70% ice cold ethanol (add ethanol and mix them a few seconds gently by hands, after that centrifugate 5 min at 14 000 g, and then dumping the

ethanol; repeat the above step again).

12. Dump the ethanol absolutely and dry the pellet 30-60 min in the speedvac (Concentrator 5301 – Eppendorf, Hamburg, Germany); solve the sample in 50 µl TE buffer (1 x TE buffer PH 8) until it completely solved (at least 12 h).
13. Measure concentration and quality of the samples with a nanodrop; quantify the samples in 1% agarose gel; and the last step is quantifying the samples with a PCR.

5.2 Primers list of *MYB14* used for TA Cloning and Gateway®-Cloning for this study

Name	Primer sequence 5'-3'
Full length of Hoe29	Sense: 5'- -3' CTACTGACGTGCACTAGCCT Antisense: 5'- -3' GCAGAGTGAAAGTGCAACACG
Full length of Ke83	Sense: 5'- -3' CTACTGACGTGCACTAGCCT Antisense: 5'- -3' GCAGAGTGAAAGTGCAACACG
Full length of Augster Weiss	Sense: 5'- -3' CTACTGACGTGCACTAGCCT Antisense: 5'- -3' GCAGAGTGAAAGTGCAACACG
Promoter of Hoe29	Sense: 5'- -3' GGGGACAAGTTTGTACAAAAAAGCAGGCTTCCTACTGACGTGCACTAGCCT Antisense: 5'- -3' GGGGACCACTTTGTACAAGAAAGCTGGGTCTTTTTCTTCTCTATGTAAGGATTTGA
Promoter of Ke83	Sense: 5'- -3' GGGGACAAGTTTGTACAAAAAAGCAGGCTTCCTACTGACGTGCACTAGCCT Antisense: 5'- -3' GGGGACCACTTTGTACAAGAAAGCTGGGTCTTTGAGACTGAGACACCCTTG
Promoter of Augster Weiss	Sense: 5'- -3' GGGGACAAGTTTGTACAAAAAAGCAGGCTTCCTACTGACGTGCACTAGCCT Antisense: 5'- -3' GGGGACCACTTTGTACAAGAAAGCTGGGTCTTTGAGACTGAGACACCCTTG

5.3 PCR reaction for amplifying the full length of *MYB14* and *MYB15*

5.3.1 Pipetting instructions

Component	Volume / 20 µl reaction
H_2O	add to 20 µl
5 x Phusion HF Buffer	4 µl
10 mM dNTPs	0.4 µl
20 µM Forward primer	0.4 µl
20 µM Reverse primer	0.4 µl
Template DNA	20-200 ng/20 µl
5 M Betaine	2 µl
DMSO	0.8 µl
Phusion™ DNA polymerase	0.1 µl

5.3.2 Cycling conditions

Cycle	Temp.	Time	Number of cycles
Initial denaturation	98 °C	30 s	1
Denaturation Annealing Extension	98 °C 56 °C 72 °C	10 s 30 s 2 min	40
Final extention	72 °C 4 °C	5 min hold	1

5.4 PCR reaction for amplifying the promoter sequence of *MYB14*

5.4.1 Reaction setup

Component	Volume / 25 µl reaction
Nuclease-Free water	add to 25 µl
5 x Q5 reaction Buffer	5 µl
10 mM dNTPs	0.5 µl
20 µM Forward primer	0.625 µl
20 µM Reverse primer	0.625 µl
Template DNA	< 1.000 ng
DNA polymerase	0.25 µl

5.4.2 Thermocycling conditions for PCR

Cycle	Temp.	Time	Number of cycles
Initial denaturation	98 °C	30 s	1
Denaturation Annealing Extension	98 °C 57 °C 72 °C	10 s 30 s 2 min	40
Final extention	72 °C 4 °C	2 min hold	1

5.5 TA cloning technology

5.5.1 Ligation protocol

1. Briefly centrifuge the pGEM®-T Easy vector tubes to collect contents at the bottom of the tube.

2. Set up ligation reactions as described below. Vortex the 2 x Rapid Ligation Buffer vigorously before each use. Use 0.5 ml tubes known to have low DNA binding capacity.

Reagents	Volume
2x Rapid Ligation Buffer, T4 DNA Ligase	5 µl
pGEM®-T Easy vector (50 ng)	1 µl
A-tailing PCR product	3 µl
T4 DNA Ligase (3 Weiss units/µl)	1 µl
Final volume	10 µl

3. Mix the reactions by pipetting. Incubate the reactions overnight at 4 °C for the maximum number of transformants.

5.5.2 Transformation protocol

1. Prepare two LB / 100 µg/ml ampicillin / 0.5 mM IPTG / 80 µg/ml X-Gal plates for each ligation reaction. Equilibrate the plates to room temperature.

2. Centrifuge the ligation reactions briefly. Add 2 µl of each ligation reaction to a sterile 1.5 ml reaction tube on ice.

3. Place the frozen 50 µl aliquots of chemo Competent DH 5α cells on ice (about 5 min). Mix the cells by gently flicking tube.

4. Carefully transfer 50 µl of cells to the ligation reaction tubes from Step2. Gently flick the tubes and incubate on ice for 30 min.

5. Transfer cells for exactly 45 sec to 42°C. DO NOT SHAKE. Immediately return the tubes to ice for exactly 2 min.

6. Add 950 µl room temperature LB medium to the ligation reaction transformations and incubate for 1.5 h at 37°C with shaking (600 rpm).

7. Spin down the cells at 3,000 rpm for 1 min, remove 800 µl LB and re-suspend cells gently.

8. Plate 50 µl and 150 µl on LB-Agar plates with ampicillin/PTG/X-Gal plates.

9. Incubate the plates overnight (16-24 hours) at 37°C. Select white colonies.

For more information concerning the TA-cloning technology, refer to the manual "pGEM®-T and pGEM®-T Easy Vector Systems" (promega: http://www.promega.com).

5.6 Gateway® recombination reactions technology

The Gateway® technology (Invitrogen Corporation, Paisley, UK) uses the bacteriophage site-specific lambda recombination system to facilitate transfer of heterologous DNA sequences between vectors (Hartley *et al*., 2000). The components of the lambda recombination sites (*att* sites) are modified to improve the specificity and efficiency of the system (Bushman *et al*., 1985).

Two recombination reactions constitute the basis of this technology:

1. BP reaction: Facilitates recombination of an *attB* substrate (attB-PCR product) with an *attP* substrate (called "donor vector") to create an *attL*-containing entry clone. This reaction is catalysed by BP Clonase™ Ⅱ enzyme mix (Invitrogen).

2. LR reaction: Facilitates recombination of an *attL* substrate (called "entry clone") with an *attR* substrate (called "destination vector") to create an *attB*-containing expression clone. This reaction is catalysed by LR Clonase™ Ⅱ enzyme mix (Invitrogen).

The presence of the *ccdB* gene within this system allows negative selection of the donor and destination vecotors in E. coli following recombination and transformation. The ccdB protein interferens with E.coli DNA gyrase (Bernard and Couturier, 1992), thereby inhibiting growth of most *E.coli* strains. When recombination occurs (i.e. between an attB-PCR product and a donor vector or between an entry clone and a destination vector), the *ccdB* gene is replaced by the gene of interest. Cells that take up unreacted vectors carrying the *ccdB* gene or by-product molecules retaining the *ccdB* gene will fail to grow. This allows high-efficiency recovery of the desired clones. For more information

concerning the Gateway® technology, refer to manual "Gateway® Technology with Clonase™ Ⅱ" (Invitrogen; http://www.invitrogen.com). This summary of the Gateway® technology was taken with from the doctoral thesis of Dr. Jan Maisch (Botanical Institute I, KIT, Karlsruhe; Maisch, 2007).

5.7 The promoter region of *MYB14* and *MYB15* in Hoe29, Ke83 and Augster Weiss

5.7.1 The promoter sequence of *MYB14*

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Hoe29          CTACTGACGTGCACTAGCCTCTTTCTTTGACCCCTTCACACCATCGATGCTAAATTCCAA 60
Ke83           CTACTGACGTGCACTAGCCTCTTTCTTTGACCCCTTCACACCATCGATGCTAAATTCCAA 60
Augster Weiss  CTACTGACGTGCACTAGCCTCTTTCTTTGACCCCTTCACACCATCGATGCTAAATTCCAA 60
Reference      CTACTGACGTGCACTAGCCTCTTTCTTTGACCCCTTCACACCATCGATGCTAAATTCCAA 60
               ************************************************************

Hoe29          GCAGCCCAAATACTTCAACAAATGTGAACTGCACGTGTACACTCTCACACTCGTGTCCAT 120
Ke83           GCAGCCCAAATACTTCAACAAATGTGAACTGCACGTGTACACTCTCACACTCGTGTCCAT 120
Augster Weiss  GCAGCCCAAATACTTCAACAAATGTGAACTGCACGTGTACACTCTCACACTCGTGTCCAT 120
Reference      GCAGCCCAAATACTTCAACAAATGTGAACTGCACGTGTACACTCTCACACTCGTGTCCAT 120
               ************************************************************

Hoe29          TTTGTGAATATGGTATTAGGTGTTGTGTTAGGGTCTAATTTTGGGTCGAGTTAAGAGAAC 180
Ke83           TTTGTGAATATGCTATTAGGTTTTGTGTTAGGGTCTAATTTTGGGTCGAGTTAAGAGAAC 180
                           MRE
Augster Weiss  TTTGTGAATATGGTATTAGGTGTTGTGTTAGGGTCTAATTTTGGGTCGAGTTAAGAGAAC 180
Reference      TTTGTGAATATGGTATTAGGTGTTGTGTTAGGGTCTAATTTTGGGTCGAGTTAAGAGAAC 180
               ************ ******** ************************************

Hoe29          ACTTATTCATCATACTTTAGCTGGATATGGAAAGTTTTTGAATATGTAATGAAGAAAAGG 240
Ke83           ACTTATTCATCATACTTTAGCTGGATATGGAAAGTTTTTGAATATGTTATGAAGAAAAGG 240
Augster Weiss  ACTTATTCATCATACTTTAGCTGGATATGGAAAGTTTTTGAATATGCAATGAAGAAAAGG 240
Reference      ACTTATTCATCATACTTTAGCTGGATATGGAAAGTTTTTGAATATGCAATGAAGAAAAGG 240
               ******************************************  ************

Hoe29          AAAGAAATTTATTTCAAATTTCATCCATTAGTATTTTTATAAATTTATTTTATTTATTAA 300
Ke83           AAAGAAATTTATTTCAAATTTCATCCATTAGTATTTTTATAAATTTATTTTATTTATTAA 300
Augster Weiss  AAAGAAATTTATTTCAAATTTCATCCATTAGTATTTTTATAAATTTATTTT--------- 291
Reference      AAAGAAATTTATTTCAAATTTCATCCATTAGTATTTTTATAAATTTATTTT--------- 291
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Hoe29          ATTTATTTTTTTTAAAGAGATTTAAATGAAAATATTTTAAGTAGAA----------TCC 349
Ke83           ATTTATTTTTTT-AAAGAGATTTAAATGAAAATATTTTAAGTAGAA----------TCC 348
Augster Weiss ----------TTTAAAGAGATTTAAATGAAAATATTTTAAGTAGAAGTGTTTAAAAATCC 341
Reference      ----------TTTAAAGAGATTTAAATGAAAATATTTTAAGTAGAAGTGTTTAAAAATCC 341
                         ** *********************************          ***

Hoe29          TCTAATAATTAAATTTTATTCTTTAAAAATTAAAATTTATATATATAAAATATATTTATA 409
Ke83           TCTAATAATTAAATTTTATTTTTTAAAAATTAAAATTTATATATATAAAATATATTTATA 408
                                    TATA-box
Augster Weiss TCTAATAATTAAATTTTATTCTTTAAAAATTAAAATTTATATATATAAAATATATTTATC 401
Reference      TCTAATAATTAAATTTTATTCTTTAAAAATTAAAATTTATATATATAAAATATATTTATC 401
               ******************* *************************************

Hoe29          GGATGTAACCGATAAAATTTGAAATATGTGGCGACTTTATATATTTACATTCAGGTGATG 469
Ke83           GGATGTAACCGATAAAATTTGAAATATGTGGCGACTTTATATATTTACATTCAGGTGATG 468
Augster Weiss GGATGTAACCGGTAAAATTTGAAATATGTGACGATTTTATATATTTACATTAGGGTGGTG 461
Reference      GGATGTAACCGGTAAAATTTGAAATATGTGACGATTTTATATATTTACATTAGGGTGGTG 461
               *********** ***************** *** ***************  **** **

Hoe29          TTTATTTTGCGATTGAATAGAAAAAA-TCAAAATATTTGATTTTTTTTAATTCAATTAAA 528
                                                    TATA-box CAAT-box
Ke83           TTTATTTTTCGAT---------------------------------------------- 481
Augster Weiss TTTATTTTTTGATTGAATAGAAAAAAATTAAAATATTTGATTTTTCT-AATTTAACTAAA 520
Reference      TTTATTTTTTGATTGAATAGAAAAAA-TCAAAATATTTGATTTTTCT-AATTTAACTAAA 519
               ********  ***

Hoe29          AATAACTCTTTGACATCGTTCAATATAACTAAACTGAACTAATTATTAATAGTTTTAGTT 588
         3-AF1 binding site                                      TATA-box
Ke83           ------------------------------------------------------------
Augster Weiss AATAACTTGTTCACATCGTTCAATATAACTAAACTGAATTTATTATTAATAGATTCAATT 580
Reference      AATAACTTGTTCACATCGTTCAATATAACTAAACTGAATTTATTATTAATAGATTCAATT 579

Hoe29          CAATTATGTTGAAAAATATAAACATGTTACTTTTAATTGAATAAAAAAAATCAAATATTT 648
             CAAT-box                          CAAT-box         CAAT-box
Ke83           -AATTATGTTGAAAAATATAAATATGTTACTTTTAACTGAATAAAAAAAGTCAAATATTT 540
               as-2-box                                          CAAT-box
Augster Weiss TAGTTATATTGAAGAATATAAACGGATTACTTTTAACTGAATATAAAAAATTAAGTATTT 640
Reference      TAGTTATATTGAAGAATATAAACGGATTACTTTTAACTGAATATAAAAAATTAAGTATTT 639
                * **** ***** ********    ********** ****** ***** * ** *****

Hoe29          TAATTTTTTATATTCAATACAAAAACAAACACCATTTACATCTCAAGATTATCAATGAAA 708
                     TATA-box   TATA-box                  circadian
Ke83           TAATTTTTTATATTCAATACAAAAACAAACATCATTTACAACTCAAGATTATCAATGAAA 600
                     TATA-box   TATA-box                  circadian
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Augster Weiss TAATTTTTCATATTCAATAAAAAAACAAACATCACTTAAGATTCA-GATTATCAATGAAA 699
Reference     TAATTTTTCATATTCAATAAAAAAACAAACATCACTTAAGATTCA-TATTATCAATGAAA 698
              ******** ********** ********** ** ***    ***  *************

Hoe29         AACTCAAAATTAAAATATTTCTGCATATATTCTAATGCACCGCCTTAAGATGAGCCGGTT 768
Ke83          AACTCAAAATTAAAATATTTCTGCATATATTCTAAAGCACCGCCTTAAGATGAGCCGGTT 660
Augster Weiss AACTCAAAATTAAAATATTTCTGCATATATTCTAAGGCACCGCCTTAAGATGAGCCTGTT 759
Reference     AACTCAAAATTAAAATATTTCTGCATATATTCTAAGGCACCGCCTTAAGATGAGCCTGTT 758
              ********************************** ******************** ***

Hoe29         ATTCTATTAAAGAGATAATAACGAATTTGAAAAGGCAGAAAAGGAAAATACCAAGAAGGA 828
Ke83          ATTCTATTAAAGAGATAATAACGAATTTGAAAAGGCAGAAAAGGAAAATACCAAGAAGGA 720
Augster Weiss ATTCTATTAAAGAGATAATAACGAATTTGAAAAGGCAGAAAAGGAAAATACCAAGAAGGA 819
Reference     ATTCTATTAAAGAGATAATAACGAATTTGAAAAGGCAGAAAAGGAAAATACCAAGAAGGA 818
              ************************************************************

Hoe29         CTTGGGGATATTGAACGTCACATTCATAGGGATCGCCTTGCAGAAGAAAACAAAAACAAG 888
Ke83          ATTGGGGATATTGAACGTCACATTCATAGGGATCGCCTTGCAGAAGAAAACAAAAACAAG 780
Augster Weiss TTTGGGGATATTGAACGTCACATTCATAGGGATCACCTTGCAGAAGAAAACAAAAACAAG 879
Reference     TTTGGGGATATTGAACGTCACATTCATAGGGATCACCTTGCAGAAGAAAACAAAAACAAG 878
              ******************************** ************************

Hoe29         ATGAAATTCATTCCGGGTAAGTTATTATATAGCAAGTTGGTGCGTTAATTTGCCAAAGTT 948
Ke83          ATGAAATTCATTCCGGGTAAGTTATTATATAGCAAGTTGGTGCGTTAATTTGCCAAAGTT 840
Augster Weiss ATGAAATTCATTCCGGGTAAGTTATTATATAGCAAGTTGGTGCGTTAATTTGCCAAAGTT 939
Reference     ATGAAATTCATTCCGGGTAAGTTATTATATAGCAAGTTGGTGCGTTAATTTGCCAAAGTT 938
              ************************************************************

Hoe29         GGTCACAAGTTTCATTAAAATAATAATAATAATAATAATAATAGGAAAGAAAGAAGGAAA 1008
                                                  TATA-box
Ke83          GGTCACAAGTTTTATTAAAATAATAATAATAATAATA------GGAAAGAAAGAAGGAAA 894
Augster Weiss GGTCACAAGTTTCATTAAAATAATAATAATAATAATA------GGAAAGAAAGAAGGAAA 993
Reference     GGTCACAAGTTTCATTAAAATAATAATAATAATAATA------GGAAAGAAAGAAGGAAA 992
              ************ ************************      *****************

Hoe29         AGAAAAATTCTTGAACTCAAATGTAAAATATCTGAACATGCCCCAATTAATGGCCATGCT 1068
Ke83          AGAAAAATTCTTGAACTCAAATGTAAAATATCTGAACACGCCCCAATTAATGGCCATG-T 953
Augster Weiss AGAAAAATTCTTGAACTCAAATGTAAAATATCTGAACATGCCCCAATTAATGGCCATGCT 1053
Reference     AGAAAAATTCTTGAACTCAAATGTAAAATATCTGAACATGCCCCAATTAATGGCCATGCT 1052
              ************************************ ****************** *

Hoe29         AGTTCAAGGAAAAGAAAAACCCCACGTTTTATTTGACCAATAAACAAACACTCGTGTCAT 1128
Ke83          AGTTCAAGGAAAAGAAAAACCCCACGTTTTATTTGACCAATAAACAAACACTCGTGTCAT 1013
Augster Weiss AGTTCAAGGAAAAGAAAAACCCCACGTTTTATTTGACCAATAAACAAACACTCGTGTCAT 1113
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Reference     AGTTCAAGGAAAAGAAAAACCCCACGTTTTATTTGACCAATAAACAAACACTCGTGTCAT 1112
              ************************************************************

Hoe29         CCAGTGAGGCGGTTCCTAGCAATTGTGGGCTAAAAAGGATATGCCTTTTTATTTTCTTTT 1188
Ke83          CCAGTGAGGCGGTTCCTAGCAATTGTGGGCTAAAAAGGATATGCCTTTTTATTTTCTTTT 1073
Augster Weiss CCAGTGAGGCGGTTCCTAGCAATTGTGGGCTAAAAAGGATATGCCTTTTTATTTTCTTTT 1173
Reference     CCAGTGAGGCGGTTCCTAGCAATTGTGGGCTAAAAAGGATATGCCTTTTTATTTTCTTTT 1172
              ************************************************************

Hoe29         TTTCTTCTTTTTTTCTAAATTCTATCAACAGTTGTTTGTCCTATCTGCAAATTGCAGCAG 1248
        5UTR Py-rich stretch                    GATA-motif
Ke83          TTT----------TCTAAATTCTATCAACAGTTGTTTGTACTATCTGCAAATTGCAGCAG 1123
Augster Weiss TTT----------TCTAAATTCTATCAACAGTTGTTTGTACTATCTGCAAATTGCAGCAG 1223
Reference     TTT----------TCTAAATTCTATCAACAGTTGTTTGTACTATCTGCAAATTGCAGCAG 1222
              ***          ************************ ********************

Hoe29         CTGGACTCCTCATTATAAATACCCGCTCATGGGCTTCAAATCGGTTTGAGCTTGGGACAT 1308
Ke83          CTGGACAGC--ACTATAAATACCCGCTCATGGGCTTCAAATCGGTTTGAGCTTGGGACAT 1181
Augster Weiss CTGGACTCCTCATTATAAATACCCGCTCATGGGCTTCAAATCGGTTTGAGCTTGGGACAT 1283
Reference     CTGGACTCCTCATTATAAATACCCGCTCATGGGCTTCAAATCGGTTTGAGCTTGGGACAT 1282
              ******  *  *  ********************************************

Hoe29         CGACAGAGAACACAG---------------------TCTCAAATCCTTACATAGAGAAGA 1347
                                                                TCA-element
Ke83          CAAGAGAGACCACAGAGATAAACAAGGGTGTCTCAGTCTCAAATCCTTACATAGAAAAGA 1241
Augster Weiss CAAGAGAGAACACAGAGATAAACAAGGGTGTCTCAGTCTCAAATCCTTACGTAGAAAAGA 1343
Reference     CAAGAGAGAACACAGAGATAAACAAGGGTGTCTCAGTCTCAAATCCTTACGTAGAAAAGA 1342
              * * ***** *****                     ************** **** ****

Hoe29         AAAA 1351
Ke83          AAAA 1245
Augster Weiss AAAA 1347
Reference     AAAA 1346
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Note: *TATA box*: core promoter element around -30 of transcription start.

CAAT box: common cis-acting element in promoter and enhancer regions.

circadian: cis-acting regulatory element involved in circadian control.

Ke83

MRE: MYB binding site involved in light responsiveness.

as-2-box: involved in shoot-specific expression and light responsiveness.

Hoe29

3-AF1 binding site: light responsive element.

5UTR Py-rich stretch: cis-acting element conferring high transcription levels.

GATA-motify: part of a light responsive element.

TCA-element: cis-acting element involved in salicylic acid responsiveness.

5.7.2 The promoter sequence of *MYB15*

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Hoe29          GCCAAGGACTTGACTTGGAAAATGGCTGACCAGTCATTTTTCATTCATCTTATTGTTCTA 60
Ke83           GCCAAGGACTTGACTTGGAAAATGGCTGACCAGTCATTTTTCATTCATCTTATTGTTCTA 60
Augster Weiss GCCAAGGACTTGACTTGGAAAATGGCTGACCAGTCATTTTTCATTCATCTTACTGTTCTA 60
Reference      GCCAAGGACTTGACTTGGAAAATGGCTGACCAGTCATTTTTCATTCGTCTTATTGTTCTA 60
               ********************************************* ***** *******

Hoe29          AGTTGCAAGCTATTGCCCTTGCCTGCTTCCCGGATCCTCCCAACTTACATTCCTTTTACA 120
Ke83           AGTTGCAAGCTATTGCCCTTGCCTGCTTCCCGGATCCTCCCAACTTACATTCCTTTTACA 120
Augster Weiss AGTTGCAAGCTATTGCCCTTGCCTGCTTCCCGGATCCTCCCAACTTACATTCCTTTTACA 120
Reference      AGTTGCAAGCTATTGCCCTTGCCTGCTTCCCGGATCCTCCCAACTTACATTCCTTTTACA 120
               ************************************************************

Hoe29          CCCAAGTAACCTCAGTCAACAAATGTGTACTCTCCCCCATCACTTTCATTGGCTACTGGA 180
Ke83           CCCAAGTAACCTCAGTCAACAAATGTGTACTCTCCCCCATCACTTTCATTGGCTACTGGA 180
Augster Weiss CCCAAGTAACCTCAGTCAACAAATGTGTACTCTCCCCCATCACTTTCATTGGCTACTGGA 180
Reference      CCCAAGTAACCTCAGTCAACAAATGTGTACTCTCCCCCATCACTTTCATTGGCTACTGGA 180
               ************************************************************

Hoe29          GTCAAATTATGATCCTCTTCTTTGACATACATGCCTTGTGTATCAGACTTGAGATGCTCT 240
Ke83           GTCAAATTATGATCCTCTTCTTTGACATACATGCCTTGTGTATCAGACTTGAGATGCTCT 240
Augster Weiss GTCGAATTATGATCCTCTTCTTTGACATACATGCCTTGTGTAACAGACTTGAGATGCTCT 240
Reference      GTCAAATTATGATCCTCTTCTTTGACATACATGCCTTGTGTATCAGACTTGAGATGCTCT 240
               *** ********************************** *****************

Hoe29          TCTTTTTTTTTTTTTTTCAAATTTTTGAAATCTTTATCAAAAAATACATTTTAAAAATGC 300
Ke83           TCTTTTTTTTTTTTTTTCAAATTTTTGAAATCTTTATCAAAAAATACATTTTAAAAATGC 300
Augster Weiss TCTTTTTTTTTTTTTTTCAAATTTTTTAAATCTTTATAAAAAAATACATTTTAAAAATGC 300
Reference      TCTTTTTTTTTTTTTTTCAAATTTTTGAAATCTTTATCAAAAAATACATTTTAAAAATGC 300
               ************************* ********** **********************

Hoe29          TTCTTAAAATATAATTTTAAATCATTTTCAAGAGTAAAGTTATATTTAAAAATTTAAAAA 360
Ke83           TTCTTAAAATATAATTTTAAATCATTTTCAAGAGTAAAGTTATATTTAAAAATTTAAAAA 360
Augster Weiss TTCTTAAAATATAATTTTAAATCATTTTCAAGAGTAAAGTTATATTTAAAATTTTAAAAA 360
Reference      TTCTTAAAATATAATTTTAAATCATTTTCAAGAGTAAAGTTATATTTAAAAATTTAAAAA 360
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                ************************************************** ********

Hoe29           TAAAATTAATTTTTCGAATTTATTCTAATTTAAATAAACGTTGAACAATCAAAATATCAT 420
Ke83            TAAAATTAATTTTTCGAATTTATTCTAATTTAAATAAACGTTGAACAATCAAAATATCAT 420
Augster Weiss   TAAAATTAATTTTTTGAATTTATTCTAATTTAAATAAAATTTGAAGAATCAAAATACCAT 420
Reference       TAAAATTAATTTTTCGAATTTATTCTAATTTAAATAAACGTTGAACAATCAAAATATCAT 420
                ************** ********************** ***** ********** ***

Hoe29           TTTTAGACTATCAAGTTAAACTTTGAACTCATAATAATAATATATACAGGTTGAAAAAAA 480
Ke83            TTTTAGACTATCAAGTTAAACTTTGAACTCATAATAATAATATATACAGGTTGAAAAAAA 480
Augster Weiss   TTTTAGACTATCAAGTTAAACTTTGAACTCATAATAATAATATATAGGGGTTGAAAAAAA 480
Reference       TTTTAGACTATCAAGTTAAACTTTGAACTCATAATAATAATATATACAGGTTGAAAAAAA 480
                ******************************************  ************

Hoe29           GAGAGAAAAATATTAAAATAATCCAATCAATCTTGGAATTTACGATCCGGAAACATGTGA 540
Ke83            GAGAGAAAAATATTAAAATAATCCAATCAATCTTGGAATTTACGATCCGGAAACATGTGA 540
Augster Weiss   GAGAGGAAAAGATTAAAATAATCCAATCAATCTTGGAATTAACGATCCGGAAACATGTGA 540
Reference       GAGAGAAAAATATTAAAATAATCCAATCAATCTTGGAATTTACGATCCGGAAACATGTGA 540
                ***** **** **************************** ******************

Hoe29           CTTATTTTTGTCGGGACCATATATGATATGTCACGTTAAGGTTACTTTTTCTAATTTCAA 600
Ke83            CTTATTTTTGTCGGGACCATATATGATATGTCACGTTAAGGTTACTTTTTCTAATTTCAA 600
Augster Weiss   CTTATTTTTCTCGGGACCATATATGATATGTCACGTTAAGGTTACTTTTTCTAATTTCAA 600
Reference       CTTATTTTTGTCGGGACCATATATGATATGTCACGTTAAGGTTACTTTTTCTAATTTCAA 600
                ********* ************************************************

Hoe29           AAGTGTATGCACAATTTTTCCACGGTGCATGGCCAACTCCTGCACTTGGAATCAATAGCC 660
Ke83            AAGTGTATGCACAATTTTTCCACGGTGCATGGCCAACTCCTGCACTTGGAATCAATAGCC 660
Augster Weiss   AAGTGTATGCACAATTTTTCCACGGTGCATGGCCAACTCCTGTACTTGGAATCAATAGCC 660
Reference       AAGTGTATGCACAATTTTTCCACGGTGCATGGCCAACTCCTGCACTTGGAATCAATAGCC 660
                ****************************************** *****************

Hoe29           AGGGCTTATGGAGGTTCATGACTCACGAGGATCATAAATTATTATTTTAATTAATTAACA 720
Ke83            AGGGCTTATGGAGGTTCATGACTCACGAGGATCATAAATTATTATTTTAATTAATTAACA 720
Augster Weiss   AGGGCTTATGGAGGTTCATGACTCACGAGGATCATAAATTATTATTTTAATTAATTAACA 720
Reference       AGGGCTTATGGAGGTTCATGACTCACGAGGATCATAAATTATTATTTTAATTAATTAACA 720
                ************************************************************

Hoe29           GTTCCTGTCCTAAAATTAAAAAATATTTATATATAAAAAAATATTAGAATTTATTCAGTT 780
Ke83            GTTCCTGTCCTAAAATTAAAAAATATTTATATATAAAAAAATATTAGAATTTATTCAGTT 780
Augster Weiss   GTTCCTGTCCTAAAATTAAAAAATATTTATATATAAAAAAATATTAGAATTTATTCAGTT 780
Reference       GTTCCTGACCTAAAATTAAAAAATATTTATATATAAAAAAATATTAGAATTTATTCAGTT 780
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Hoe29          GTTTTTGTTTTTAAATATAAAAAACTGTTTATAAAAATTTATAAAAATTATTTTTCATCA 840
Ke83           GTTTTTATTTTTAAAAATAAAAAACTGTTTATAA----------AAATTATTTTTCATCA 830
Augster Weiss  GTTTTTATTTTTAAAAATAAAAAACTGTTTATAAAAATTTATAAAAATTATTTTTCATCA 840
Reference      GTTTTTGTTTTTAAATATAAAAAACTGTTTATAAAAATTTATAAAAATTATTTTTCATCA 840
               ****** ******** ******************          ****************

Hoe29          GTTGTTTTTTGAAATTAACTTTTAAAAATAAAGAACAATTTTTAAAAAATAAATTAAAAT 900
Ke83           GTTGTTTTTTGAAATTAACTTTTAAAAATAAAGAACAATTTTTAAAAAATAAATTAAAAT 890
Augster Weiss  GTTGTTTTTTGAAATTAACTTTTAAAAATAAAGAACAATTTTTAAAAAATAAATTAAAAT 900
Reference      GTTGTTTTTTGAAATTAACTTTTAAAAATAAAGAACAATTTTTAAAAAATAAATTAAAAT 900
               ************************************************************

Hoe29          TATTTTTATCTATTTTTAAAAATAAAAGAAAAATAGGATTCAACCTTATCATATTTTTAG 960
Ke83           TATTTTTATCTATTTTTAAAAATAAAAGAAAAATAGGATTCAACCTTATCATATTTTTAG 950
Augster Weiss  TATTTTTATCTATTTTTAAAAATAAAAGAAAAATAGGATTCAACCTTATCATATTTTTAG 960
Reference      TATTTTTATCTATTTTTAAAAATAAAAGAAAAATAGGATTCAACCTTATCATATTTTTAG 960
               ************************************************************

Hoe29          AAAATTATTTTTAATAATTAATTTTATTTTTTAATTTTTAATTTAAAAATAATTTTAAAA 1020
Ke83           AAAATTATTTTTAATTATTAATTTTATTTTTTAATTTTTAATTTAAAAATAATTTTAAAA 1010
Augster Weiss  AAAATTATTTTTAATAATTAATTTTATTTTTTAATTTTTAATTTAAAAATAATTTTAAAA 1020
Reference      AAAATTATTTTTAATAATTAATTTTATTTTTTAATTTTTAATTTAAAAATAATTTTAAAA 1020
               ************** ******************************************

Hoe29          AACAAGTTTAAGAGAAAAAGGCAAACAAACCTTAAATTTTTGTTATGTTTTTCAAATTTA 1080
Ke83           AACAAGTTTAAGAGAAAAAGGCAAACAAACCTTAAATTTTTGTTATGTTTTTCAAATTTA 1070
Augster Weiss  AACAAGTTTAAGAGAAAAAGGCAAACAAACCTTAAATTTTTGTTATGTTTTTCAAATTTA 1080
Reference      AACAAGTTTAAGAGAAAAAGGCAAACAAACCTTAAATTTTTGTTATGTTTTTCAAATTTA 1080
               ************************************************************

Hoe29          TTCGGTTGTTTTTTGTTTTTAAAAGTAAAAAACTATTTTTAAAAATTTGTAATCTTGTTT 1140
Ke83           TTCGGTTGTTTTT-GTTTTTAAAAGTAAAAAACTATTTTTAAAAATTTGTAATCTTGTTT 1129
Augster Weiss  TTCGGTTGTTTTT-GTTTTTAAAAGTAAAAAACTATTTTTAAAAATTTGTAATCTTGTTT 1139
Reference      TTCGGTTGTTTTTTGTTTTTAAAAGTAAAAAACTATTTTTAAAAATTTGTAATCTTGTTT 1140
               ************* ********************************************

Hoe29          CATCAATTGTTTTTTGAAATTAACTTTTAAAAATAAAGAACAAA-TTTTAAAAATAAATT 1199
Ke83           CATCAATTGTTTTTTGAAATTAACTTTTAAAAATAAAGAACAAT-TTTTAAAAATAAATT 1188
Augster Weiss  CATCAATTGTTTTTTGAAATTAACTTTTAAAAATAAAGAACAAAATTTTAAAAATAAATT 1199
Reference      CATCAATTGTTTTTTGAAATTAACTTTTAAAAATAAAGAACAAA-TTTTAAAAATAAATT 1199
               ****************************************  ***************

Hoe29          AAAATTATTTTCATCTATTTTTTTTTTTTTAAAAAAAAATTGGGATCC-GTCTTATCATAT 1258
Ke83           AAAATTATTTTCATCTATTTTTTTTTTTTAAAAAAAAAATTGGGATCC-GTCTTATCATAT 1247
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Augster Weiss AAAATTATTTTCATCTATTTTTTTTTA-AAAAAAAAAATTGGGATCCTGTCTTATCATAT 1258
Reference     AAAATTATTTTCATCTATTTTTTTTTTTTAAAAAAAAATTGGGATCC-GTCTTATCATAT 1258
              *************************    ***************** ************

Hoe29         TTTTAGAAATTTAATTTTAAAAATTACTTTTATTTTTTAACTTTTAATTTAAAAATAATT 1318
Ke83          TTTTAGAAATTTAATTTTAAAAATTACTTTTATTTTTTAACTTTTAATTTAAAAATAATT 1307
Augster Weiss TTTTAGAAATTTAATTTTAAAAATTACTTTTATTTTTTAACTTTTAATTTAAAAATAATT 1318
Reference     TTTTAGAAATTTAATTTTAAAAATTACTTTTATTTTTTAACTTTTAATTTAAAAATAATT 1318
              ************************************************************

Hoe29         TTTTAAAACAAGTTTAAGAGAAGAAGACAAACAAGCCTTAAATTTTTCCTATGTTTTTCA 1378
Ke83          TTTTAAAACAAGTTTAAGAGAAGAAGACAAACAAGCCTTAAATTTTTCCTATGTTTTTCA 1367
Augster Weiss TTTTAAAACAAGTTTAAGAGAAGAAGACAAACAAGCCTTAAATTTTTCCTATGTTTTTCA 1378
Reference     TTTTAAAACAAGTTTAAGAGAAGAAGACAAACAAGCCTTAAATTTTTCCTATGTTTTTCA 1378
              ************************************************************

Hoe29         ACGTTTTACAATTTATTAAAATCGGTTTTTAAGAATTTTTACATCCAATGTAGAATCTAG 1438
Ke83          ACGTTTTACAATTTATTAAAATCGGTTTTTAAGAATTTTTACATCCAATGTAGAATCTAG 1427
Augster Weiss ACGTTTTACAATTTATTAAAATCGGTTTTTAAGAATTTTTACATCCAATGTAGAATCTAG 1438
Reference     ACGTTTTACAATTTATTAAAATCGGTTTTTAAGAATTTTTACATCCAATGTAGAATCTAG 1438
              ************************************************************

Hoe29         TAATCTACTGTTTGCTTTTATATTAAAAATAGAAATAGGAAAGCCTATTGAAGCCAACCT 1498
Ke83          TAATCTACTGTTTGCTTTTATATTAAAAATAGAAATAGGAAAGCCTATTGAAGCCAACCT 1487
Augster Weiss TAATCTACTGTTTGCTTTTATATTAAAAATAGAAATAGGAAAGCCTATTGAAGCCAACCT 1498
Reference     TAATCTACTGTTTGCTTTTATATTAAAAATAGAAATAGGAAAGCCTATTGAAGCTAACCT 1498
              ********************************************************* *****

Hoe29         AACTCATTCAAACTTGAAGAGAGAGAGAGAG--AAAATATCCTCCTTCCGGGAAATTGCA 1556
Ke83          AACTCATTCAAACTTGAAGAGAGAGAGAGAG--AAAATATCCTCCTTCCGGGAAATTGCA 1545
Augster Weiss AACTCATTCAAACTTGAAGAGAGAGAGAGAG--AAACTATCCTCCTTCCGGGAAATTGCA 1556
Reference     AACTCATTCAAACTTGAAGAGAGAGAGAGAGAGAAAATATCCTCCTTCCGGGAAATTGCA 1558
              ******************************  *** **********************

Hoe29         GCAAAGTTGCTTTCTTGGACTACTAATCTTCCAGCACATTAACCCTCGTGGCTTTGGAAT 1616
Ke83          GCAAAGTTGCTTTCTTGGACTACTAATCTTCCAGCACATTAACCCTCGTGGCTTTGGAAT 1605
Augster Weiss GCAAAGTTGCTTTCTTGGACTACTAATCTTCCAGCACATTAACCCTCGTGGCTTTGGAAT 1616
Reference     GCAAAGTTGCTTTCTTGGACTACTAATCTTCCAGCACATTAACCCTCGTGGCTTTGGAAT 1618
              ************************************************************

Hoe29         AGTACGTCTATATGGTAGGATAAATACCCACACGACAACAGGGAAAATCACCTCCAGATT 1676
Ke83          AGTACGTCTATATGGTAGGATAAATACCCACACGACAACAGGGAAAATCACCTCCAGATT 1665
Augster Weiss AGTACGTCTATATGGTAGGATAAATACCCACACGACAACAGGGAAAATCACCTCCAGATT 1676
Reference     AGTACGTCTATATGGTAGGATAAATACCCACACGACAACAGGGAAAATCACTTCCAGATT 1678
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Hoe29           GAGGAAAGCAGAGCTAGCCAGAGGTTAGAGTAGTGGTGTTCCTATTGGAGAACA 1730
Ke83            GAGGAAAGCAGAGCTAGCCAGAGGTTAGAGTAGTGGTGTTCCTATTGGAGAACA 1719
Augster Weiss GAGGAAAGCAGAGCTAGCCAGAGGTTAGAGTAGTGGTGTTCCTATTGGAGAACA 1730
Reference       GAGGAAAGCAGAGCTAGCCAGAGGTTAGAGTAGTGGTGTTCCTATTGGAGAACA 1732
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5.8 Primers list for genetic inheritance in breeding plants

Name	**Primer sequence 5'-3'**
Hoe29 x Augster Weiss	Sense: 5’- -3’ CAGCAGCTGGACTCCTCATT Antisense: 5’- -3’ ATGGAGCTCTCCCCATTTTT
Ke83 x Augster Weiss	Sense: 5’- -3’ TTTGAAATATGTGGCGACTTT Antisense: 5’- -3’ CTCATCTTAAGGCGGTGC

Publication

Duan D, Halter D, Baltenweck R, Tisch C, Tröster V, Kortekamp A, Hugueney P, Nick P. 2015. Genetic diversity of stilbene metabolism in *Vitis sylvestris*. Journal of Experimental Botany **66**, 3243-3257.