University of Neuchâtel
Institute of Botany
Laboratory of Biochemistry

β-aminobutyric acid-induced resistance in grapevine against downy mildew (*Plasmopara viticola*)

PhD thesis presented by

Mollah Md. Hamiduzzaman

Neuchâtel, Switzerland 2005

Université de Neuchâtel unine

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Mollah Md. HAMIDUZZAMAN

UNIVERSITE DE NEUCHATEL

FACULTE DES SCIENCES

La Faculté des sciences de l'Université de Neuchâtel,
sur le rapport des membres du jury

Mme B. Mauch-Mani (directrice de thèse),
MM. R. Tabacchi, J.-M. Neuhaus,
A. Pugin (Dijon) et F. Mauch (Fribourg)

autorise l'impression de la présente thèse.

Neuchâtel, le 27 septembre 2005

La doyenne:

M. Rahier

Faculté des Sciences
■ Rue Emile-Argand 11 ■ CP 2 ■ CH-2007 Neuchâtel
■ Téléphone : +41 32 718 21 00 ■ Fax : +41 32 718 21 03 ■ E-mail : secretariat.sciences@unine.ch ■ www.unine.ch

Acknowledgements

All praises are addressed to the Almighty Allah who enabled me to complete this dissertation.

I sincerely express my heartfelt respects and deep sense of gratitude to Dr. Brigitte Mauch-Mani for her supervision, precious suggestions, constructive criticisms and giving me the opportunity to learn about plant-oomycete interactions and induced resistance.

I am grateful to Prof. Jean-Marc Neuhaus for his scholastic guidance, valuable suggestions and constructive discussions.

I want to specially thank Dr. Gabor Jakab for his valuable advice, kind help in molecular biology techniques, constructive discussions and correcting my thesis.

I sincerely express my gratitude to Prof. Alain Pugin, Prof. Felix Mauch, Prof. Raphael Tabacchi, who kindly accepted to be the jury of my PhD thesis. I am thankful to them for their constructive suggestions and co-operation.

I express gratitude to my mother Hazera Bewa and my father late Alhajj Sefatullah Mollah (died on 29 January 2005 during my PhD study) for their inspiration and continued support.

I express my heartfelt lot of love to my wife Monira Sultana for her encourage, inspiration, on going support, mental help and to my sweet heart son Fahim Zaman.

I take the opportunity to thank my brother and sisters, all other relatives, friends and well wishers for their indirect help and rendering inspiration.

I express my special thank to Julien Bernard Jean-Denis for helping me in HPLC technique. I am thankful to all other members of the laboratory of Biochemistry at the University of Neuchâtel, especially to Jurriaan, Laurent, Romain, Michael, Svenn, Ronald, Sophie for their scientific discussions and help me in many occasions.

I would like to dedicate

this dissertation to

the departed soul of my father

Abstract

Plants respond to pathogen attack by activating signaling networks based on molecules such as salicylic acid (SA) and jasmonic acid (JA) followed by the accumulation of pathogenicity-related proteins, phytoalexins and other defense compounds. We used ß-aminobutyric acid (BABA), a non-protein amino acid to induce resistance in grapevine (*Vitis vinifera*) against downy mildew (*Plasmopara viticola*) and investigated the possible mechanisms responsible for the observed protective effect. Sporulation of *P. viticola* was reduced in BABA-treated seedlings as well as in leaf discs in both a susceptible variety (Chasselas) and a resistant variety (Solaris). Comparing different inducers, the best protection was achieved with BABA followed by JA, while BTH and abscisic acid (ABA) did not significantly increase the resistance. The potentiated accumulation of callose and lignin in BABA-treated plants was decreased by the co-application of DDG (an inhibitor of callose production) and AIP (an inhibitor of phenyl alanin ammonium lyase) respectively. *PR-1*, a marker gene for the SA pathway was expressed in both water and BABA-treated leaf discs, whereas *PR-4* and *LOX-9*, markers for the JA pathway, showed potentiated expression in BABA-treated plants following *P. viticola* infection. Expression of *PR-4* and *LOX-9* in BABA-treated plants was decreased by the co-application of ETYA (a LOX inhibitor). The older, necrotic leaves as well as leaves infected with powdery mildew (*Uncinula necator*) accumulated defense-related genes in grapevine. Necrosis was observed in BABA-treated plants following infection with *P. viticola*. Accumulation of different phenolics and stilbenes in BABA-treated plants increased with time following infection with *P. viticola*. We used the HPLC technology to assess the involvement of stilbenes in BABA-induced resistance (BABA-IR). In the inoculation zones of leaf discs, accumulation of *trans*-resveratrol was much higher in BABA-treated than in control plants (in both Chasselas and Solaris) following infection with *P. viticola.* Outside of the inoculation

zone accumulation levels of stilbenes were low. In seedlings, however, accumulation of *trans*-resveratrol was primed in both necrotic and non-necrotic zones, while *trans*-epsilon-viniferin was only primed in necrotic zones of BABA-treated plants. Furthermore, expression of the stilbene synthase gene (*STS-1*) was also higher in JA-treated plants than in BTH- and ABA-treated plants. The induction of *STS-1* was observed in BABA-treated plants upon infection with *P. viticola*. Our results suggest that BABA-IR involves priming of the JA signal transduction pathway and leads to a potentiated accumulation of callose, lignin, resveratrol and viniferins in grapevine following infection with downy mildew.

Abbreviations

ABA	abscisic acid
aba1-5	abscisic acid deficient 1-5
abi4-1	abscisic acid insensitive 1
AIP	2-aminoindan-2-phosphonic acid
ARR	age-related resistance
Atvsp	Arabidopsis thaliana vegetative storage protein
AOS	active oxygen species
BABA	β-amino butyric acid
BABA-IR	β-amino butyric acid-induced resistance
BTH	benzothiadiazole
Chit3	Chitinase 3
CCoAOMT	S-adenosyl-L-methionine:trans-caffeoyl-coenzyme A 3-O-methyltransferase
coi1	coronatine insensitive 1
Col-0	columbia 0
CS	callose synthase
DDG	2-deoxy-D-glucose
DNA	deoxyribonucleicacid
eds4	enhance disease susceptibility 4
ein2	ethylene insensitive 2
etr1	ethylene response 1
ETYA	5, 8, 11, 14–Eicosatetraynoic acid
Gluc1	β-1,3-glucanase 1
GSL5	glucan synthase-like 5

GST1	glutathion-S-transferase 1
GUS	β-glucuronidase
HPLC	high-performance liquid chromatography
HR	hypersensitive reaction
HSR1	hypersensitive response factor 1
INA	2,6-dichloroiso-nicotinic acid
ISR	induced systemic resistance
JA	jasmonic acid
jar1	jasmonic acid resistant 1
LOX	lipoxygenase
MJ	methyl jasmonate
mRNA	messenger ribonucleic acid
NahG	salicylate hydroxylase gene
npr1	no pathogenesis-related protein 1
nim1	no immunity 1
OSM-1	osmotin 1
pad4	phytoalexin deficient 4
PAL	phenylalanine ammonia-lyase
PDF1.2	plant defensin 1.2
PGPR	plant growth promoting rhizobacteria
PIN1	serine proteinase inhibitor 1
pmr4	powdery mildew resistant 4
PR-protein	pathogenesis-related protein
rRNA	ribosomal ribo-nucleic acid

SA	salicylic acid
SAR	systemic acquired resistance
STS-1	stilbene synthase 1
TMV	tobacco mosaic virus
UV	ultra violet
VCH3	Vitis acidic class III chitinase
VCHIT1b	Vitis acidic class I chitinase
Vst-1	vitis stilbene synthase 1
WCS417r	*Pseudomonas fluorescens* strain 417

Contents

CHAPTER 1

General introduction

Grapevine (*Vitis vinifera*) is the most widely cultivated fruit crop in the world and the most important in economic terms. In contrast to other fruits, grapes are not only used as a fruit (fresh fruit, fruit juice, dried fruit, etc.), but are the basis for the production of high added-value products such as wine and spirits. Because of these multiple uses, grapevine has been a part of human culture since the establishment of agricultural societies, thousands of years ago. Today, grapevine is more than an economically valuable crop. It is an important component of Western societies by providing substantial employment through a large number of diverse jobs, and is in many cases associated with the national culture or lifestyle.

An important crop in Switzerland, grapevine is affected by several fungal and oomycete diseases such as grey mould (*Botrytis cinerea*), powdery mildew (*Uncinula necator*) and downy mildew (*Plasmopara viticola*). Downy mildew causes considerable damage to grapevine in Switzerland as well as all over the world. *P. viticola* is a biotrophic oomycete,

belonging to the family Peronosporaceae. Generally, *P. viticola* attacks the leaves, flowers, tendrils, stems as well as young berries. *P. viticola* completes its life cycle within 6 days.

Modern agriculture is characterized by very high productivity. Its major problem is the indiscriminate use of chemicals such as fungicides, bactericides and nematicides which not only raises production costs, but also leads to the contamination of soil and water resources, causing environmental pollution which threatens the future of agriculture. To alleviate this problem, major advances have been accomplished to better understand the plant's own defense reactions against pathogens.

A new technology for plant disease control is based on the activation of the plant's own defense system with the aid of low molecular weight synthetic molecules. Compounds such as salicylic acid (SA), 2,6-dichloroiso-nicotinic acid (INA) and benzo(1,2,3)-thiadiazole-7-carbothionic acid-S-methyl ester (BTH)) are able to induce systemic acquired acquired resistance (SAR) in a variety of plants against a wide range of microbial pathogens (Sticher et al., 1997). DL-3-amino-n-butanoic acid or DL-β-aminobutyric acid (BABA), a non-protein amino acid, is able to potentiate signal transduction pathways in different plants against a broad spectrum of microbial pathogens (Zimmerli et al., 2000; Conrath et al., 2002; Ton and Mauch-Mani, 2004).

Plant resistance to pathogen infection

Plant resistance can be broadly defined as the plant's ability to suppress or slow down the damaging activity of the pathogen (Agrios, 1997). Resistance operates at different levels and can accordingly be subdivided into different classes (Mauch-Mani, 2002). The most effective type of resistance is non-host resistance. A plant species not affected by certain pathogens is

considered as a non-host for those pathogens and the resistance expressed as non-host resistance. This resistance protects the plant against pathogen infection and is expressed when a plant comes into contact with different microbial organisms. According to Mauch-Mani (2002), general resistance confers a partial and quantitative protection also known as field or horizontal resistance and is usually not race-specific. The rate of disease progress is reduced compared to a susceptible plant while plants showing quantitative resistance upon infection. On the other hand, gene-for-gene resistance is based on the specific interaction between the products of avirulence genes in the pathogen and resistance gene in the host and is race-specific (Flor, 1971). Several researchers have been working to identify and isolate resistance (R) genes in grapevine. Recently, Di Gaspero and Cipriani (2003) reported that Nucleotide Binding Site/Leucine-Rich Repeat (NBS-LRR) and Serin/Threonine Kinase (STK) genes, two of the known classes of resistance (R) genes, occur in grapevine in large multigene families. The newly-bred Regent is a resistant cultivar of grapevine that has field-resistance to multiple fungal diseases inherited as polygenic traits, and Lemberger is a susceptible cultivar. Fischer et al. (2004) studied the genetic traits of the F1 population from the cross between these cultivars of grapevine (Regent x Lemberger) and identified a quantitative trait locus for resistance to downy and powdery mildew diseases. Resistance mechanisms in grapevine are complex and different constitutive substances are involved. Resistant grapevine accumulates phytoalexins (Langcake, 1981) and lignin (Dai et al., 1995) and deposits callose (Kortekamp et al., 1997) following fungal attack. We have used two varieties of grapevine: Chasselas is highly susceptible to *P. viticola*, whereas Solaris shows significant resistance. The resistance of Solaris involves callose accumulation (Gindro et al., 2003). However, there are in plants additional types of resistance to pathogenic microorganisms that are based on both constitutive and inducible resistance defense reactions as will be discussed in the following paragraphs.

Constitutive defense mechanisms

The first line of defense a pathogen will encounter during plant infection consists of structural preformed barriers such as the cuticle and cell wall. The cuticle layer represents the first barrier for the pathogen to penetrate directly through the cell wall (Kerstiens, 1996). Chemical constitutive barriers have also been described. Saponins occur as preformed chemical compounds in plant tissues. The resistance of oats to the root-infecting fungus *Gaeumannomyces graminis* var. *tritici* has been associated with the triterpinoid avenacin saponins which were found in oat roots (Maizel et al. 1964). The typical preformed, constitutive defenses are the structural barriers like waxes, cutin, suberin and cellulose (Jeandet et al., 2002).

Inducible defense mechanisms

Plants activate various defense mechanisms upon recognition of a pathogen. For examples, plants protect themselves with additional structural barriers such as formation of papillae, tyloses and abscission zones (Agrios, 1997). Lignification can improve plant resistance to fungal penetration (Hijwegen 1963). Antifungal phytoalexins are not present in healthy plants but are synthesized after pathogen attack or stress as part of the plant defense response and are restricted to the cells surrounding the infection site (Müller and Börger 1940, Paxton 1981).

Induced resistance

Induced resistance depends either on defensive compounds that are produced as a result of the inducing treatment, and/or on a faster and stronger activation of plant defense mechanisms upon infection with a pathogen or stress. Different types of induced resistance such as

systemic acquired resistance (SAR), induced systemic resistance (ISR) and β-aminobutyric acid-induced resistance (BABA-IR) will be discussed in the following paragraphs.

Systemic acquired resistance

When a plant is attacked by a pathogen causing necrotic lesions or a hypersensitive reaction (HR) it is often observed that non-infected distant plant parts become resistant to many different pathogens. This phenomenon is called systemic acquired resistance. One of the first modifications in plant metabolism after SAR induction is a local and systemic accumulation of endogenous salicylic acid (SA; Malamy et al., 1990; Metraux et al., 1990). Exogenous application of SA induces *PR* proteins and leads to an enhanced level of resistance (Uknes et al., 1992). Some of these proteins (chitinases, glucanase, taumatin) showed antimicrobial properties (Mauch et al., 1988), so that they are commonly used as markers for SAR. Application of functional analogues of SA, such as INA and BTH induced the same set of *PR* genes as biological induction of SAR. INA and BTH are effective against the same spectrum of plant pathogens (Uknes et al., 1992; Ryals et al., 1996; Lawton et al., 1996). For example, INA-treated cucumber shows enhanced incorporation of cell wall-associated phenolics only upon challenge with *Colletotrichum lagenarium* (Siegrist et al., 1994). To elucidate the SAR activity, the expression of chitinases was studied in SA- and BTH-pre-treated grapevine upon infection with *P. viticola*. Busam et al. (1997) showed that expression of VCH3 (acidic class III chitinase) was preferentially induced compared to VCHIT1b (basic class I chitinase) following infection in grapevine.

Rhizobacteria-mediated induced systemic resistance

Selected non-pathogenic, root-colonizing *Pseudomonas* bacteria have been shown to induce resistance in all parts of the plant. This type of induced resistance, called rhizobacteria-mediated induced systemic resistance (ISR; Van Loon et al., 1998), has been observed in several plant species (Van Peer et al., 1991; Duijff et al., 1998). Several of these non-pathogenic rhizobacterial strains are capable of stimulating plant growth and are therefore called plant growth promoting rhizobacteria (PGPR; Kloepper et al., 1980). In *Arabidopsis*, the *Pseudomonas fluorescens* strain WCS417r has been used to induce ISR against fungal and bacterial pathogens, demonstrating, as for SAR, a broad-spectrum of activity (Pieterse et al., 1996; Van Wees et al., 1997). Interestingly, WCS417r-mediated ISR functions independently of SA and *PR* gene activation (Pieterse et al., 1996; Van Wees et al., 1997), but requires components of the JA and ethylene responses. WCS417r failed to trigger ISR in *jar1* (Staswick et al., 1992) and *etr1* (Bleecker et al., 1988) mutants showing that the ISR pathway requires sensitivity to JA and ethylene (Pieterse et al., 1998). Although there are many differences between the mechanisms underlying the SAR and ISR pathways, there seems to be a common signalling component. Pieterse et al. (1998) demonstrated that the *Arabidopsis npr1* mutant not only failed to express SAR, but was also impaired in WCS417r-induced ISR. Thus, NPR1 is required for both SA-dependent SAR and JA- or ethylene-dependent ISR expression.

Chemical SAR activators

To be considered as a chemical SAR activator, neither the chemical compound nor its metabolites should demonstrate direct antibiotic activity *in vitro* or *in planta*. Additionally, the compound has to be efficient against the same broad spectrum of pathogens as biological SAR, with similar protection at phenotypic and molecular levels (Kessmann et al., 1994). Compounds such as SA, INA and BTH are able to induce systemic acquired resistance in a

variety of plants against a wide range of microbial pathogens without possessing direct antimicrobial activity *in vitro* or *in planta*. SA, INA and BTH are functionally related, sharing the activation of similar genes (coding for PR-proteins) in the plant. Both INA and BTH act independently of the presence of SA. They are active in monocotyledonous as well as dicotyledonous plants and normally require about 2 days for gene activation (Kessmann et al., 1994; Ryals et al., 1996; Sticher et al., 1997). They fail to induce SAR in *npr1* mutants of *Arabidopsis* (Delaney, 1997), which proves again that they work only by activating the plant's own defense mechanisms.

Salicylic acid (SA)

SA is a key endogenous component for the activation of SAR (Hammond-Kosack and Jones, 1996; Durner et al., 1998). Exogenous application of SA is sufficient to mimic pathogen-induced SAR with concomitant expression of *PR* genes (Ryals et al., 1996). The use of transgenic tobacco and *Arabidopsis* expressing a bacterial salicylate hydroxylase gene (*NahG*), the product of which converts SA into inactive catechol (Gaffney et al., 1993; Delaney et al., 1994), demonstrates the requirement for SA as a signal in SAR. Indeed, NahG plants fail to develop SAR when infected with pathogens or after exogenous application of SA.

While SA is required in the systemic tissue for the expression of *PR* genes, it has been proposed that it is not the only primary long-distance signal for the induction of SAR (Willits and Ryals, 1998). Indeed, wildtype scions grafted onto NahG rootstocks were still able to express SAR, indicating that a SAR-inducing signal different from SA is probably responsible for resistance (Vernooij et al., 1994). Recently, a lipid transfer protein was implicated in the modulation of the SAR mobile signal (Maldonado et al., 2002). However, SA seems to be

necessary for SAR expression in non-infected tissue (Vernooij et al., 1994; Willits and Ryals, 1998).

In connection with defense reactions in plants the molecular mechanisms of age-related resistance (ARR) has received some attention. Kus and colleagues (2002) reported that the ability to accumulate SA is necessary for the ARR-response in Arabidopsis against *P. syringae* pv. *tomato*. Munne-Bosch and Alegre (2002) demonstrated the relationship between oxidative stress and plant aging, and suggested that the oxidative stress increases progressively in chloroplasts as plants age.

INA (2,6-dichloroiso-nicotinic acid)

INA and BTH have been shown to activate the same spectrum of resistance as SAR does with concomitant activation of SA-dependent *PR* genes (Uknes et al., 1992; Lawton et al., 1996). Consequently, they are considered as synthetic chemical activators of SAR. INA and its methyl ester are efficient agents against a wide spectrum of pathogens, ranging from viruses and bacteria to fungi (Uknes et al., 1992; Kogel et al., 1994; Nielsen et al., 1994). INA directly induces the expression of SAR genes and demonstrates a low *in vitro* antifungal activity (Ward et al., 1991). In tobacco and in *Arabidopsis*, INA is still fully active in NahG-expressing plants, demonstrating that it can replace or operate downstream of SA (Vernooij et al., 1994). One major hurdle with commercial applications of INA-like compounds is their phytotoxic effects on certain crops (Sticher et al., 1997).

Benzo(1,2,3)-thiadiazole-7-carbothionic acid-S-methyl ester (BTH)

BTH also shows SAR-like activities in a number of plants such as wheat, rice, tobacco and *Arabidopsis* (Sticher et al., 1997). Like INA, BTH has almost no direct antifungal activity and

leads to the activation of the same SAR genes as SA (Friedrich et al., 1996; Gorlach et al., 1996; Lawton et al., 1996). Since, neither INA nor BTH protect *nim1*/*npr1* mutants, it has been postulated that both compounds act through a common signalling cascade downstream of SA perception (Delaney et al., 1995; Lawton et al., 1996).

Jasmonic acid (JA)

JA, a product of the lipoxygenase (LOX) pathway, has been proposed to be a signal transducer of defense reactions in plants. Rickauer et al. (1992) reported that methyl jasmonate (MJ) induced accumulation of proteinase inhibitors in tobacco cells. Lipoxygenase has been reported to be induced in various plants after pathogen attack (Fournier et al. 1993 ; Melan et al. 1993). JA and its methyl ester MJ are well established as signals in plant defense responses, and in other hormonal roles such as stomatal functioning and tendril coiling (Sticher et al., 1997). As it is also involved in tuber formation, JA controls both storage of carbohydrates, and their utilisation in defense products, and may thus control the ratio of carbon-rich phenolics to nitrogen-rich proteinase inhibitors (Creelman and Mullet, 1997). JA and MJ are synthesized from membrane lipids, along with other molecules including phytodienoic acid, arachidonic acid and traumatin (Farmer, 1994), all involved in defense responses. Defensins are one of the main products of the JA signalling pathway. They are small cystein-rich peptides that are also known in the immune system of animals and have been isolated from plants (Broekart, 1995). SA antagonises the production of JA (Penninckx et al., 1996) meaning that the production of PR proteins can override the production of proteinase inhibitors when the plant is under pathogen attack and hence producing SA.

Abscisic acid (ABA)

The phytohormone ABA is involved in the regulation of many physiological stresses in plants. Exogenous application of ABA prior to inoculation increases the susceptibility of tomato, Arabidopsis or grapevine to various pathogens (Audenaert et al., 2002; Mohr and Cahill, 2003; Lu et al., 2001). Exogenous application of ABA prior to inoculation induces resistance against necrotrophic pathogens in Arabidopsis (Ton and Mauch-Mani, 2004) and regulates plant responses to abiotic stresses (Xiong et al., 2002). In contrast, the susceptibility of tomato plants to *Botrytis cinerea* was enhanced by ABA treatment (Audenaert et al., 2002) and a high ABA concentration was observed in grapevine shoots with Pierce's disease symptoms compared to healthy shoots (Lu et al., 2001).

β-aminobutyric acid (BABA)

BABA has biological activity in both animals and plants. In animals, BABA can act as a partial agonist of the glycine receptor (Schmieden and Betz, 1995). In plants, local treatment with BABA has been shown to protect them against various pathogens. First observations were reported some 40 years ago, when protection of peas against the oomycete *Aphanomyces euteiches* was demonstrated (Papavizas and Davey, 1963; Papavizas, 1964).

Cohen et al. (1999) observed that BABA protects grape leaves from the mildew when applied after infection. Even when applied 48h after inoculation, protection was achieved compared to the control. It has been speculated that BABA deteriorates the fungus-penetrated host cells so that translocation of nutrients into the haustoria is blocked, thus preventing further mycelial growth and sporangia production (Steiner and Schönbeck, 1997). Zimmerli et al. (2000) observed that the protective effect of BABA is due to a potentiation of natural defense mechanisms against biotic and abiotic stresses. Local treatments with BABA systemically

protect tomato and potato (against *Phytophthora infestans*; Cohen, 2000; Si-Ammour et al., 2003) and tobacco (against *Peronospora tabacina*; Cohen et al., 1994).

Moreover, recent reports show that BABA also protects plants against a nematode and a virus (Oka et al., 1999; Siegrist et al., 2000), demonstrating the broad range of activity of this chemical. The possible direct toxicity of BABA on many plant pathogens was repeatedly tested *in vitro* and *in vivo* by different research groups (Jakab et al., 2001). A direct antimicrobial activity of this chemical was never observed (Cohen et al., 1994; Li et al., 1996; Sunwoo et al., 1996; Cohen et al., 1999; Hong et al., 1999; Tosi et al., 1999). Experiments with ^{14}C-labelled BABA clearly show that it is not metabolized in tomato (Cohen and Gisi, 1994) or in *Arabidopsis* (Jakab et al., 2001), ruling out the involvement of a BABA-metabolite as antimicrobial compound in the plant. Thus BABA-mediated resistance is most likely based on the activation of host resistance mechanisms. Nevertheless, the mode of action of this chemical is still a matter of controversy: some studies report induction of *PR* genes after BABA treatment (Cohen et al., 1994), while in another study the result was different (Cohen, 1994). BABA-IR of Arabidopsis against *Pseudomonas syringae* and *Botrytis cinerea* was impaired in transgenic NahG plants as well as in *npr1* mutant (Zimmerli et al., 2000, 2001). These authors showed that *PR-1* gene expression was faster in BABA-treated plants than in non-treated control upon infection with pathogens, although BABA did not induce expression of the *PR-1* gene directly. Ton and Mauch-Mani (2004) demonstrated that BABA-IR against necrotrophic fungi is not based on SA-, JA-, or ethylene-dependent defense mechanisms nor on enhanced accumulation of camalexin, but is regulated by a ABA-dependent signalling pathway. Ton et al. (2005) reported that mutants of Arabidopsis that are impaired in BABA priming are also impaired in the expression of induced resistance. This clearly shows that priming has a genetic basis.

Histopathology and accumulation of phytoalexins

Plants produce an enormous array of secondary metabolites, and it is commonly accepted that a significant part of this chemical diversity serves to protect plants against microbial pathogens (Dixon, 2001). These low molecular weight plant substances are classified as phytoanticipins, which are compounds that are present constitutively, or phytoalexins, the levels of which increase strongly in response to microbial invasion (Kuc and Rush, 1985). The phytoalexins are lipophilic substances, accumulating at the site of infection and inhibiting the development of fungi and bacteria. BABA was reported to increase PAL activity and to induce resistance against late blight in potato leaf discs (Newton et al., 1997). The induction of lignin accumulation by BABA in HR-responding mesophyll cells hints at the possible involvement of enzymes like PAL and peroxidase. Dai and colleagues (1995) observed that flavonoids play a key role in a resistant grape species (*Vitis rotundifolia*), and that resveratrol at an early stage, and flavonoids and lignin at a later stage may play a role in restricting the growth of *P. viticola* in intermediate resistant species (*V. rupestris*). The necrotic lesions in BABA-treated grapevine against *P. viticola* revealed the accumulation of lignin-like deposits (Cohen et al., 1999). In contrast, Kortekamp et al. (1997) reported that callose accumulation was observed in tolerant cultivars (Orion and Phoenix) at a late stage (5 days after inoculation) indicating that mechanisms different from callose deposition are involved in the defense against *P. viticola*.

Phytoalexins were originally recognized as antifungal compounds produced by plants upon pathogen attack (Cruickshank, 1963). The accumulation of stilbenes was induced in grape by chemicals, UV irradiation or by infection with *P. viticola* or *B. cinerea* in grapevine (Barlass et al., 1987; Langcake 1981; Langcake and Pryce, 1976, 1977a). *trans*-resveratrol has been

suggested to be a precursor of viniferins (Langcake, 1977; Langcake and Pryce, 1977b; Langcake et al., 1979; Pryce and Langcake, 1977) whose synthesis varies with environmental conditions and *Vitis* species.

Signals and cross-talk between defense pathways in plants

SA, JA and ethylene are plant signal molecules playing key roles in the regulation of defense responses against pathogens and herbivores. Reducing the biosynthesis of, or sensitivity to either JA or ethylene can also render plants more susceptible to pathogens and insects. For example, ethylene-insensitive tobacco plants lose their ability to resist to the soil-borne oomycete *Pythium sylvaticum* (Knoester et al., 1998). Similarly, Arabidopsis mutants affected in JA biosynthesis or signalling are more susceptible to *Pythium irregulare* (Staswick et al., 1998) and insect herbivory (McConn et al., 1997; Stout et al., 1999). The jasmonic acid pathway induces defensin synthesis, leads to induction of osmotin, proline rich glycoproteins, synthesis of phytoalexins (Wasternack, 1997) and proteinase inhibitors (Sticher et al., 1997). SA, JA and ethylene are each involved in controlling basal resistance against different pathogens. These regulators control and potentiate the activity of distinct defense pathways to single or multiple stresses (Reymond and Farmer, 1998; Glazebrook, 1999; Pieterse and Van Loon, 1999). Penninckx and co-workers (1998) showed that treatment with ethylene or MJ of *coi1* and *ein2* (Guzman and Ecker, 1990) mutants respectively, did not cause the accumulation of PDF1.2 mRNA as in wild type Col-0. Moreover, they showed that exogenous application of MJ and ethylene had a synergistic effect on the expression level of PDF1.2. Negative interactions have also been reported. Genes activated by JA/ethylene are hyperinducible in the SAR defective plants NahG and *npr1* (Penninckx et al., 1996; Clarke et al., 1998), as well as *eds4, pad4* mutants with a reduced SA level displayed a higher PDF1.2 mRNA accumulation

after treatments with MJ and/or rose bengal (Vaijayanti et al., 2000), supporting the idea that SA interferes with JA-dependent signalling. In different plants, it has been demonstrated that SA, INA and BTH suppress JA-dependent defense gene expression (Doherty and Bowles, 1988; Peña-Cortes et al., 1993; Niki et al., 1998). Conversely, Lawton and colleagues (1995) showed that JA and ethylene have also effects on the level of SA-induced gene expression in Arabidopsis. The plant hormone ABA regulates different signalling pathways involved in plant responses to abiotic stresses. Genetic analysis has demonstrated that the ABA signalling pathway interacts antagonistically with the ethylene signalling pathway (and vice versa) to modulate plant development (Beaudoin et al., 2000; Ghassemian et al., 2000). Xiong and Yang (2003) observed the antagonistic interaction between biotic and abiotic stress in rice. In contrast, others (Park et al., 2001; Mengiste et al., 2003, Chini et al., 2004) suggested that biotic and abiotic stress responses also share common components.

BABA applied as a foliar spray induced SA accumulation in tobacco (Siegrist et al., 2000) and tomato (Jeun et al., 2000) suggesting that BABA operates via the SA-signalling pathways. However, tobacco plants expressing the salicylic acid-hydroxylase gene (*NahG*), and therefore impaired in the salicylic acid signalling pathway of SAR, were protected by BABA against *Peronospora tabacina* (Cohen, 1994) but not against TMV (Siegrist et al., 2000), indicating pathogen-specific mechanisms of defense. Ton and Mauch-Mani (2004) reported that BABA-IR against necrotrophic pathogens was compromised in the ABA-deficient mutant (*aba1-5*) and ABA-insensitive mutant (*abi4-1*). They showed that exogenous application of ABA induced callose accumulation and resistance against *Alternaria brassicicola* and *Plectosphaerella cucumarina*. More recently, Anderson et al. (2004) reported that the antagonistic interactions between multiple components of ABA and the JA-ethylene signalling

pathways modulate defense- and stress-responsive gene expression in response to biotic and abiotic stresses in Arabidopsis.

Inhibitors of different defense pathways

Just as it is important for enzymes to catalyze biological reactions, so is the ability to control and regulate enzymatic activity. This is the role of small, specific molecules and ions known as enzyme inhibitors. Inhibitors are often molecules that are similar in shape to a substrate and can thus fit into the active site of the enzyme that was intended for the substrate. Once the inhibitor occupies the active site, however, it does not allow the enzyme to catalyze the reaction. Unlike other plants (e.g. Arabidopsis), in grapevine there are no mutants related to SA, JA and ABA signal transduction pathways. To be able to further study the involvement of these defense pathways during BABA-induced resistance in grapevine, different inhibitors of defense-related enzymes were used.

To investigate the role of callose deposition in BABA-IR in grapevine against *P. viticola*, we used the callose synthase inhibitor DDG (2-deoxy-D-glucose). Nishimura et al. (2003) found that *powdery mildew resistant 4* (*pmr4*), an Arabidopsis mutant lacking pathogen-induced callose synthase, became resistant to pathogens, rather than more susceptible. This resistance was linked to a hyperactive SA response, suggesting that callose synthesis negatively regulates the SA pathway. Phenylalanine ammonia-lyase (PAL) is an enzyme of phenylpropanoid metabolism in higher plants which gives rise to a large number of compounds such as lignins, flavonoids, coumarins, stilbenes, salicylic acids (Hahlbrock and Scheel, 1989). To assess the possible involvement of lignification and SA-dependent signalling pathway in BABA-IR the effect of PAL inhibitor AIP (2-aminoindan-2-phosphonic acid) has been demonstrated. Mauch-Mani and Slusarenko (1996) reported that PAL is involved in synthesizing SA and precursors for lignification in Arabidopsis, and AIP made the plants completely susceptible to

Hyaloperonospora parasitica. To gain more insight into the involvement of the JA pathway the lipoxygenase (LOX) the inhibitor ETYA (5, 8, 11, 14-Eicosatetrayonic acid) was used. Lox inhibitors have been successfully used to inhibit wound- or elicitor-induced responses in various plants (Staswick et al. 1991; Peña-Cortes et al. 1993; Ellard-Ivey and Douglas 1996). Rickauer et al. (1997) investigated the role of LOX and jasmonate in tobacco plants infected with *Phytophthora parasitica* var. *nicotianae* by using ETYA. They suggested that an array of defense-related transcripts was induced by methyl jasmonate in tobacco, but they seemed to be regulated only partially by the LOX-catalysed jasmonate pathway.

Priming/potentiation of signal transduction pathways

Some defense responses are not directly activated during induced resistance but are induced more quickly and efficiently after challenge inoculation with a pathogen, a phenomenon referred to as "sensitization", "potentiation", or "priming" (Conrath et al., 2002). Hammerschmidt and Kuc (1995) showed that cucumber leaves exhibiting induced SAR showed a more rapid and up-regulated lignification of host cell walls in response to inoculation with *Colletotrichum lagenarium*. On the other hand, SAR induced tobacco plants overexpressed PR-10 and PAL genes when infected with TMV (Mur et al., 1996). JA has been shown to potentiate elicitor-induced accumulation of active oxygen species in cultured parsley cells (Kauss and Jeblick, 1995) whereas, ethylene potentiates the SA-dependent *PR-1* gene expression in Arabidopsis (Lawton et al., 1995). It also has been proposed that an ubiquitin-proteasome system may play a role in the potentiation process in cucumber (Becker et al., 2000).

Conrath and colleagues (2002) recently state that BABA exerts its effect on the defence capacity of plants via priming. The term "priming" was explained as the capacity to express a

faster and stronger basal defense response upon pathogen infection. Zimmerli et al. (2000) observed that BABA induced resistance in Arabidopsis against *H. parasitica* correlated with an earlier and stronger formation of callose-rich papillae, suggesting the observed resistance was based on primed callose deposition. More recently, Aziz et al. (2003) reported that β-1,3-glucan laminarin is an efficient elicitor of defense response in grapevine cells and plants against *B. cinerea* and *P. viticola*.

Outline of the thesis

The objective of the reported work was to investigate the components playing a role in the establishment of BABA-induced resistance against *P. viticola* in grapevine.

In chapter 2, the mechanisms of BABA-IR in grapevine against *P. viticola* are demonstrated. Different staining techniques were used to visualize biochemical changes at the cellular level. The reactions observed during BABA-IR in the susceptible variety (Chasselas) were compared to naturally occurring disease resistance in the resistant cultivar (Solaris). To gain more insight, we investigated the mechanisms and the involvement of signal transduction pathways in BABA-IR against *P. viticola*.

In chapter 3, the expression profile of marker genes of different signal transduction pathways in grapevine is discussed. Unlike in other plants (e.g. Arabidopsis), there were no marker genes available for SA, JA and ABA signal transduction pathways in grapevine. To be able to further study the involvement of these defense pathways during BABA-IR in grapevine, a total of 27 putative defense-related genes were tested for SA- , JA- and ABA-inducibility in detached leaves as well as in seedlings. The marker genes of different defense pathways in

grapevine were identified. The expression pattern of marker genes was tested in leaves in relation to aging, wounding, as well as downy and powdery mildew development.

In chapter 4, the involvement of phenolics and stilbenes like *trans*-resveratrol and viniferins is demonstrated in the establishment of BABA-IR in grapevine against *P. viticola*. HPLC methods were used for the quantification of phenolics. The accumulation of phenolics observed during BABA-IR in the susceptible variety Chasselas was compared to naturally occurring disease resistance in the cultivar Solaris. Leaf discs as well as seedlings (Chasselas) were used in these experiments. The toxic effect of stilbenes on the zoospores of *P. viticola* was observed. To gain more insight, we also explored the involvement of a stilbene synthase gene (*STS-1*) in BABA-IR against *P. viticola*.

In the final discussion in chapter 5, the mechanisms of BABA-IR in grapevine against *P. viticola* and the involvement of signal transduction pathways are discussed with reference to the current knowledge about plant-pathogen interactions.

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CHAPTER 2

β-aminobutyric acid-induced resistance against downy mildew in grapevine acts through the potentiation of callose formation and JA signalling

Mollah Md. Hamiduzzaman, Gabor Jakab, Laurent Barnavon, Jean-Marc Neuhaus and Brigitte Mauch-Mani

Mol. Plant-Microbe Interact. (2005) 18: 819-829

β-aminobutyric acid-induced resistance against downy mildew in grapevine acts through the potentiation of callose formation and JA signalling

Mollah Md. Hamiduzzaman, Gabor Jakab, Laurent Barnavon, Jean-Marc Neuhaus and Brigitte Mauch-Mani*

University of Neuchâtel, Institute of Botany, Department of Biochemistry, Rue Emile-Argand 11, Case Postale 2, CH-2007 Neuchâtel, Switzerland.

*For correspondence (fax: +41 32 718 2201; e-mail: brigitte.mauch@unine.ch)

Current address of L. Barnavon: INRA Dijon, Université de Bourgogne, 17, rue Sully, BP 86510, 21065 Dijon CEDEX, France

Key words: Marker gene expression, oomycete, priming of resistance, resistance inducer.

Running title: BABA-induced resistance against downy mildew in grapevine

ß-aminobutyric acid (BABA) was used to induce resistance in grapevine (*Vitis vinifera*) against downy mildew (*Plasmopara viticola*). This led to a strong reduction of mycelial growth and sporulation in the susceptible variety Chasselas. Comparing different inducers, the best protection was achieved with BABA followed by jasmonic acid (JA), while BTH (a salicylic acid (SA) analogue) and abscisic acid (ABA) treatment did not increase the resistance significantly. Marker genes for the SA- and JA-pathways showed potentiated expression patterns in BABA-treated plants following infection. The callose synthesis inhibitor 2-Deoxy-D-glucose (DDG) partially suppressed BABA- and JA-induced resistance against *P. viticola* in Chasselas. Application of the phenylalanine ammonia lyase (PAL) inhibitor AIP (2-Aminoindan-2-phosphonic acid) and the lipoxygenase (LOX) inhibitor ETYA (5, 8, 11, 14–Eicosatetraynoic acid) also led to a reduction of BABA-induced resistance (BABA-IR) suggesting that callose deposition, as well as defence mechanisms depending on phenylpropanoids and the JA pathways all contribute to BABA-IR. The similar phenotype of BABA- and JA-induced resistance, the potentiated expression pattern of JA-regulated genes (*LOX-9* and *PR-4*) following BABA treatment and the suppression of BABA-IR with ETYA suggest an involvement of the JA pathway in BABA-IR of grapevine leading to a primed deposition of callose and lignin around the infection sites.

INTRODUCTION

Grapevine (*Vitis vinifera*), the major fruitcrop worldwide, is affected by many diseases. Especially downy mildew, caused by the oomycete *Plasmopara viticola*, leads to massive damage and yield losses in this crop if no protective measures are taken. Although some grape varieties (e.g. Solaris, Regent) are resistant against downy mildew, most traditional varieties,

planted on over 90% of the wine growing area, are susceptible to this disease, necessitating an intensive use of plant protection chemicals to limit the damage in the vineyards. Since this is not compatible with a modern sustainable viticulture which aims at lowering the input of pesticides into the environment and also in view of the appearance of pesticide-resistant pathogen strains, efforts are being made to develop alternative protection strategies. One solution might be the activation of the plant's own defence system, known as induced resistance (Sticher et al., 1997). Synthetic molecules such as salicylic acid (SA), 2,6-dichloro-isonicotinic acid (INA), and benzo (1,2,3)-thiadiazole-7-carbothionic acid-S-methyl ester (BTH), are able to induce systemic acquired resistance (SAR) in a variety of plants against a wide range of microbial pathogens (Sticher et al., 1997). This type of induced resistance is characterized by various early cellular changes and an increase in salicylic acid (SA) synthesis in both primary infected and distally non-infected leaves. Moreover, SAR is typified by enhanced systemic expression of so-called SAR genes coding for PR proteins (reviewed in Ryals et al., 1996). Since some of these proteins have demonstrated antifungal activity (Mauch et al., 1988) it is thought that they contribute to protection and are therefore commonly used as markers for SAR. SAR normally requires a lapse period between treatment and inoculation (Kessmann et al., 1994) allowing for signal transduction and gene activation to take place. This is the case with SAR induction by either biological agents (e.g. TMV in N-tobacco) or chemical activators (e.g. SA, INA and BTH).

Differential expression of chitinases has been used as a characteristic factor to evaluate the SAR response in grapevine against *P. viticola* by Busam and colleagues (1997a). These authors also implied that the expression of genes of the phenylpropanoid pathway in grapevine was induced by SAR activators and suggested a role for CCoAOMT (S-adenosyl-L-

methionine:trans-caffeoyl-coenzyme A 3-O-methyltransferase) and stilbene synthase in the disease-resistance response (Busam et al., 1997b).

Jasmonic acid (JA), a product of the lipoxygenase (LOX) pathway, has been proposed to be a signal transducer of defence reactions in plants. Methyl jasmonate (MJ), e.g., has been shown to induce accumulation of proteinase inhibitors in tobacco cells (Rickauer et al., 1992) and lipoxygenases are induced in various plants following pathogen attack (Fournier et al. 1993; Melan et al. 1993). Farmer et al. (2003) proposed the existence of two pathways by which JA-related compounds can activate genes: both cyclopentenones, such as oxophytodienoic acid (OPDA), and JA activate and repress the expression of different, but overlapping sets of genes.

Another type of resistance known as induced systemic resistance (ISR) mediated by nonpathogenic, root-colonizing Pseudomonads has been observed in several plant species (Van Peer et al., 1991; Duijff et al., 1998) and shown to induce resistance in all parts of the plant (Van Loon et al., 1998). Interestingly, ISR functions independently of SA and *PR* gene activation, but requires components of the JA and ethylene responses (Pieterse et al., 1998). Indeed, *jar1* (Staswick et al., 1992) and *etr1* (Bleecker et al., 1988) mutants do not express ISR upon treatment with Pseudomonads (Pieterse et al., 1998). Thus, SAR and ISR are regulated by distinct signaling pathways.

The non-protein amino acid BABA has previously been shown to induce resistance against many different oomycetes. Local treatments of tomato, potato and tobacco with BABA led to a systemic protection against *Phytophthora infestans* and *Peronospora tabacina*, respectively (Cohen et al., 1994; Cohen, 1994). BABA was also effective in inducing resistance against downy mildew in lettuce (Pajot et al., 2001), in cauliflower (Silue et al., 2002) and Arabidopsis (Zimmerli et al., 2000). The protective effect of BABA is not restricted to

oomycetes since plants have also been shown to build up resistance towards fungi, nematodes and viruses following BABA treatment (Jakab et al, 2001, Cohen et al., 2002). Cohen et al. (1999) observed that BABA protected grape leaves from mildew infection when applied post-infectionally. It has been speculated that BABA deteriorates penetrated host cells so that translocation of nutrients into the haustoria is blocked, thus prohibiting further mycelial growth and sporangial production (Steiner and Schönbeck, 1997). A direct antimicrobial activity of this chemical has never been observed. Experiments with ^{14}C-labelled BABA clearly show that it is not metabolized in tomato (Cohen and Gisi, 1994) or in Arabidopsis (Jakab et al., 2001), ruling out the involvement of a BABA-metabolite acting as an antimicrobial compound in the plant. Thus, BABA-mediated resistance is most likely based on the activation of host resistance mechanisms. Some studies report an induction of *PR* genes after BABA treatment (Cohen et al., 1994), while others state the contrary (Cohen, 1994). Zimmerli et al. (2000) observed that the protective effect of BABA in Arabidopsis was due to the potentiation of natural defence mechanisms against biotic stresses, a phenomenon later referred to as priming (Conrath et al., 2002).

Priming is the capacity of a plant to express a faster and stronger basal defence response upon pathogen infection (Conrath et al., 2002).. Since BABA-primed resistance of Arabidopsis against *Hyaloperonospora parasitica* correlated with an earlier and stronger formation of callose-rich papillae (Zimmerli et al., 2000) it suggested that BABA-IR in this case was based on primed callose deposition. The plant hormone abscisic acid (ABA) seems to play an important role in this priming reaction (Ton and Mauch-Mani, 2004, Ton et al., 2005). However, exogenous application of ABA prior to inoculation increases the susceptibility of tomato, Arabidopsis, and grapevine to various pathogens (Flors et al., 2005).

BABA treatment also potentiated the SA regulated defence mechanisms in Arabidopsis (Zimmerli et al., 2000) contributing to the observed BABA-IR against *H. parasitica*. Mutants of Arabidopsis that are impaired in priming through BABA are also impaired in the expression of induced resistance (Ton et al., 2005). This clearly shows that BABA-induced resistance has a genetic basis. Recently, Aziz et al. (2003) reported that the β-1,3-glucan laminarin was an efficient elicitor of defence response in grapevine cells and plants against *Botrytis cinerea* and *P. viticola* and that it acted through priming.

To investigate the components playing a role in the establishment of BABA-IR against *P. viticola* in grapevine different staining techniques were used to visualize biochemical changes at the cellular level. The reactions observed during BABA-IR in the susceptible variety were compared to naturally occuring disease resistance in the resistant cultivar. The mechanisms and the involvement of different signal transduction pathways in BABA-IR against *P. viticola* were investigated. Because signalling mutants of grapevine are not available different metabolic inhibitors were used to achieve this goal.

RESULTS

BABA-treated grapevine plants show resistance against *P. viticola* and primed PR gene expression.

Seedlings of grapevine cultivar Chasselas were treated by soil drench with either water (control) or BABA (0.5mM). The inoculation with *P. viticola* was performed by dipping the seedlings in a sporangial suspension of the oomycete. Six days post inoculation heavy sporulation of downy mildew was observed on the abaxial surface of the inoculated leaves of the control plants (Fig. 1A). BABA treatment, however, highly reduced the sporulation of *P.*

viticola into restricted patches on the lower leaf surface (Fig. 1A). Using different concentrations of BABA, the disease severity was quantified at 6 and 10 days post inoculation. White clusters of sporangiophores were observed on the lower surface of the inoculated leaves covering almost 100% of the leaf surface of non-treated (control) seedlings 6 days after inoculation. At the same time point sporulation was absent in plants treated with 1, 2 or 4 mM BABA, whereas, sparse sporulation (10-20% of leaf surface) was observed in plants treated with the lowest concentration of BABA (0.5 mM, Fig. 1B). A slight increase in sporulation 10 days after inoculation was observed in plants treated with 0.5 or 1mM BABA, while 2 and 4 mM BABA still gave full protection (Fig. 1B). Light necrosis was observed in leaves of BABA-treated plants upon infection with *P. viticola*.

Expression profiling of marker genes for both the SA and JA signal transduction pathways was used to determine their involvement in the observed BABA-IR. Since no appropriate marker genes of grapevine have been characterized we determined the expression pattern of several defense-related genes after treatment with JA, BTH and ABA. *PR-1* showed a high induction after BTH treatment although it also responded to other treatments and stress conditions but to a lower extent (Fig. 2A; Jakab et al., unpublished result). *PR-4* and *LOX-9* on the other hand were highly induced by JA treatment (Fig. 2A; Jakab et al., unpublished result). Therefore, *PR-1* was selected as a marker gene for the SA pathway and *PR-4* and *LOX-9* as marker genes for JA signalling. All three marker genes of grapevine showed a potentiated expression pattern in BABA-treated plants after infection with *P. viticola* (Fig. 2B). The expression level of *PR-1* reached its maximum 24 hours after inoculation in the controls, but 13 h earlier in BABA treated seedlings. Both *PR-4* and *LOX-9* showed a potentiated expression pattern in BABA-treated plants with an increase in their expression 7 hours post inoculation and peaking at 11 hpi (Fig. 2B).

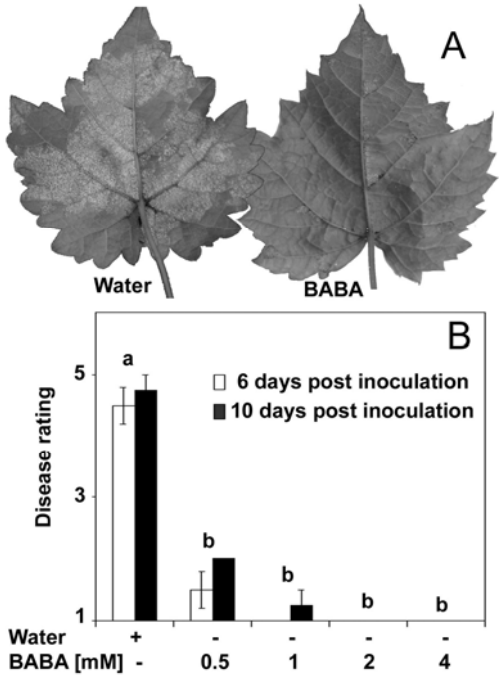

Fig. 1. Development of downy mildew (*Plasmopara viticola*) on seedlings of grapevine (Chasselas). **A**, Sporulation of *P. viticola* on the leaves 6 days after inoculation (0.5 mM BABA). **B**, Disease rating at 6 days (white bars) and 10 days (dark bars) post inoculation. Seedlings of grapevine (Chasselas) were taken at the 4-5 leaf stage. The plants were soil-drenched with water or BABA (0.5, 1, 2, 4 mM) 2 days prior to challenge inoculation with sporangia of *P. viticola* ($5x10^4$ sporangia mL^{-1}). Values presented are means ± sem (Tamhane's test ; $\alpha = 0.05$). The experiment was repeated 3 times with similar results.

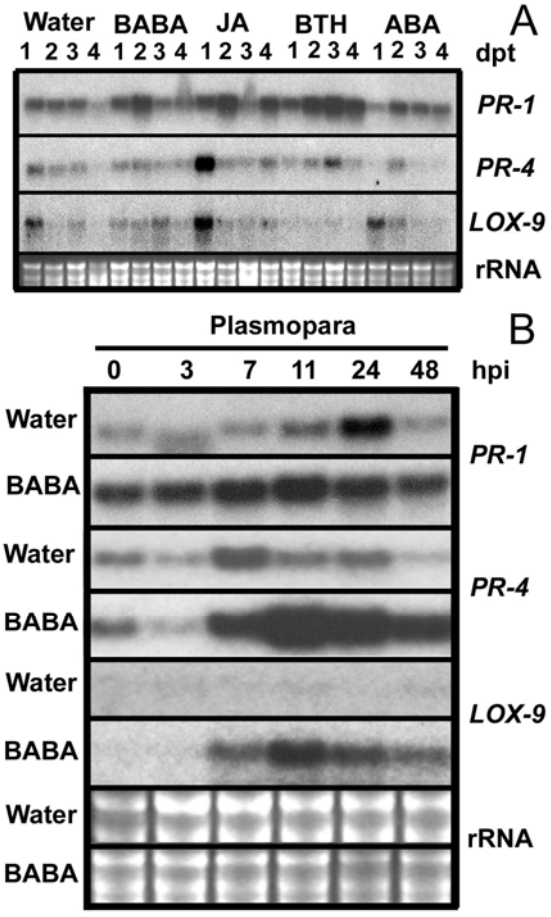

Fig. 2. Expression pattern of the *PR-1*, *LOX-9* and *PR-4* genes in seedlings of grapevine (Chasselas) upon treatment with different inducers (A) and infection with *Plasmopara viticola* (B). **A**, Inducers (BABA, 1mM; JA, 50 µM; BTH, 300 µM; ABA, 40 µM) were applied as soil drench and leaves of seedlings were collected at different time points as indicated. **B,** Seedlings were soil-drenched with water or BABA (1 mM) 2 days prior to challenge inoculation with sporangia of *P. viticola* ($5x10^4$ sporangia mL^{-1}). Inoculated leaves of seedlings were collected at different time points as indicated. Each time point represents 1 g of leaf materials (8 leaves from 4 different plants). RNA blots were hybridised with *PR-1*, *PR-4* and *LOX-9* probes. Ethidium bromide staining of the RNA gel (rRNA) was used to show equal loading. The experiment was repeated twice with similar results. dpt: days post treatment; hpi: hours post inoculation.

Development of *P. viticola* in susceptible and resistant grapevine with and without BABA treatment.

To better understand the mechanisms of BABA-induced resistance in grapevine, beside the highly susceptible cultivar Chasselas a resistant variety, Solaris, was included as a reference. Discs punched from different leaves were placed with their adaxial surface on wet Whatman paper in Petri dishes. The leaf discs were inoculated with droplets of a sporangial suspension of *P. viticola*. The sporangia released zoospores that germinated and the germ tubes penetrated into the stomata approximately 7 hours after inoculation. White sporulation of *P. viticola* could be observed by naked eye 4 days after inoculation in Chasselas (Fig. 3A). The presence of clusters of sporangiophores was confirmed with a dissecting microscope. The density of sporangiophores was very high in Chasselas and no necrosis of the plant tissue was observed (Fig. 3A). However, only very few sporangiophores appeared on BABA-treated Chasselas (Fig. 3B) and necrosis surrounded the inoculation site. In Solaris, occasionally a few sporangiophores developed and necrosis surrounded the inoculation site similarly to BABA-treated Chasselas (Fig. 3C). Sporulation was completely absent in BABA-treated Solaris and leaf discs showed a brown discoloration at the cut edge (Fig. 3D).

At the microscopic level, hyphal growth of *P. viticola* was observed at 7 hpi and spread within 3 days after inoculation in untreated Chasselas (control, Fig. 3E). In BABA-treated Chasselas, however, necrotic plant cells were observed adjacent to the hyphae (Fig. 3F). Five days post inoculation sporulation was observed in non-treated Chasselas. Several sporangiophores grew out of a single stoma in non-treated discs (Fig. 3G) but only single distorted sporangiophores bearing a low number of sporangia grew out from single stomata following BABA-treatment (Fig. 3H) and this weak sporulation was not visible macroscopically in the BABA-treated

discs. Sporangiophores were usually absent in Solaris. Occasionally , sparse sporulation was observed in non-treated Solaris but never following BABA-treatment.

Quantification of callose and lignin deposition around the infection sites.

Plant tissues stained with calcofluor aniline blue display a bright yellow-white fluorescence under UV light that corresponds to callose deposition. This fluorescence was absent in non-treated Chasselas (Fig. 3I), but could be observed in BABA-treated Chasselas (Fig. 3J) as well as in both treated (Fig. 3K) and non-treated Solaris (Fig. 3L). The fluorescence was much stronger 3 days after inoculation than after 1 day and it was stronger in treated than non-treated Solaris plants. The light blue-white fluorescence, clearly distinguishable from the yellow-white fluorescence of callose, shows the presence of oomycete structures on the leaves (Fig. 3M). Strong yellow-white fluorescence was observed mostly in stomata and in the host cells surrounding the sporangiophores in BABA-treated Chasselas as well as in both non-treated and BABA-treated Solaris. No callose deposition was visible in non-treated Chasselas within 3 days of inoculation although sometimes light yellow-white fluorescence appeared in the veins.

DDG, an inhibitor of callose synthesis (Jakab et al., 2001) was used to demonstrate the correlation between callose deposition and the observed yellow-white fluorescence in grapevine leaves. Yellow spots corresponding to callose deposition were absent in non-treated Chasselas (Fig. 3M) as well as in DDG-treated plants (Fig. 3N), whereas they were observed in high density surrounding the infected area in BABA-treated Chasselas (Fig. 3O). The callose deposition density, however, decreased with the co-application of DDG and BABA (Fig. 3P).

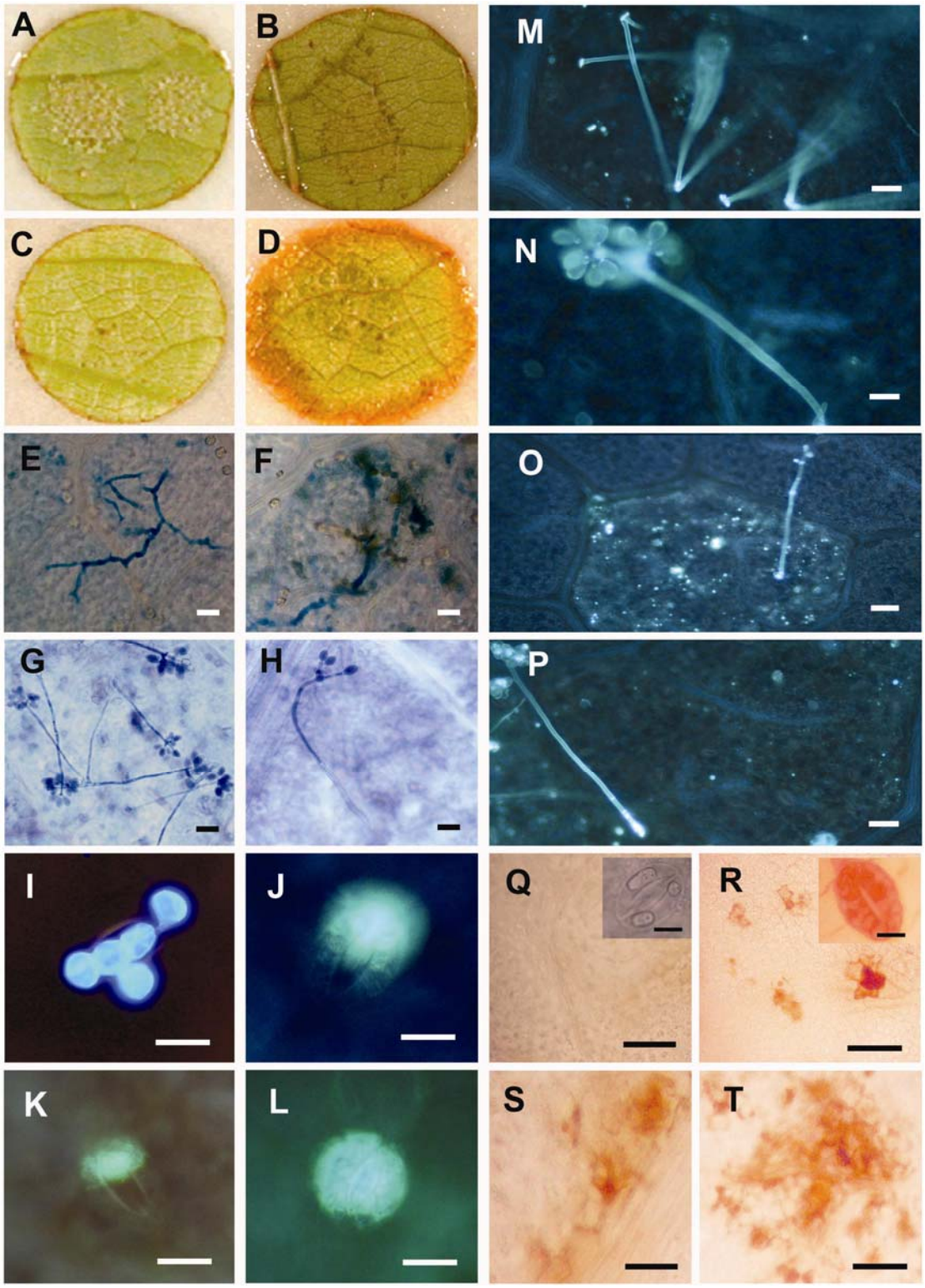
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Fig. 3. Development of necrosis, priming of callose deposition and lignification in BABA-treated grapevine following infection with *Plasmopara viticola*. **A** through **T**, leaf discs (1 cm diameter) of grapevine (all panels are Chasselas except C, D, K, L, S, and T are Solaris) were punched from the third and fourth leaves from the top of a plant. Discs were treated with water and BABA (1 mM) 24 hours prior to challenge inoculation with sporangia of *P. viticola* ($5x10^4$ sporangia mL^{-1}). The pictures were taken 5 days post inoculation (dpi) with *P. viticola* (A, B, C, and D). Leaf discs were stained with lactophenol-trypan blue and analysed by light microscopy (E, F, G, and H), or stained with calcofluor-aniline blue and analysed by epifluorescence microscopy (I, J, K, L, M, N, O, and P), or stained with phloroglucinol-HCL and analysed by light microscopy (Q, R, S, and T). **A**, non-treated control with white sporulation (Chasselas); **B**, BABA-treated Chasselas showing light necrosis at the inoculation site; **C**, non-treated Solaris; **D**, BABA-treated Solaris displaying light necrosis at the inoculation site and an increased necrotic wound reaction at the edge of the disc. **E**, intercellular hyphae of *P. viticola* in non-treated control (3 dpi); **F**, intercellular hyphae surrounded by necrosis in BABA-treated leaf tissue(3 dpi); **G**, cluster of sporangiophores (6 dpi) on non-treated control; **H**, distorted sporangiophore (6 dpi) with low number of sporangia following BABA treatment. **I** (non-treated control Chasselas), germinating zoospores (2 dpi) entering into a stoma (no callose deposition); **J** (BABA-treated Chasselas) callose deposition in the stomatal cavity, **K** (non-treated Solaris) callose deposition in the stomatal cavitiy, and **L** (BABA-treated Solaris), prominent callose deposition in the stomatal cavity. Chasselas (5 dpi): **M** (non-treated control) with numerous sporangiophore stalks visible, and **N** (callose inhibitor DDG-treated), development of sporangiophores (no callose deposition); **O** (BABA-treated), callose deposition visible as yellow-white spots surrounding a single, distorted sporangiophore; **P** (BABA and DDG-treated), decreased callose deposition visible as 36 lower number of yellow-white spots. **Q** (non-treated control Chasselas, 3dpi), no lignification (red colored tissues) visible; **R** (BABA-treated Chasselas, 3dpi), red stained lignin in tissues and in stomatal cells (inset); **S** (non-treated Solaris), and **T** (BABA-treated Solaris), both showing lignified cells (stained red) at 3 dpi. Bar = 20 μm.

Lignification in grapevine leaves was visualised by phloroglucinol-HCl staining leading to a red-coloration of lignin. Lignification was first observed 24 hours after inoculation and it increased with time. In non-treated Chasselas, no red-stained cells were observed after infection with *P. viticola* (Fig. 3Q). However, in BABA-treated Chasselas and in both non-treated and BABA-treated Solaris red-stained cells (cell walls and cytoplasm) were observed, mostly confined to the host cells surrounding the infected area (Fig. 3R, 3S, 3T, respectively) and in the guard cells of stomata (Fig. 3R, inset).

Comparison of the efficiency of different inducers of resistance against *P. viticola*.

To compare BABA-IR with BTH- and JA-induced resistance, leaf discs of plants were treated with the respective substances, inoculated and the number of sporangia were counted at 6 dpi. The highest number of sporangia was observed in non-treated control plants (Chasselas, Fig. 4A). BABA induced highest protection against *P. viticola*, followed by JA, whereas BTH treatment led to very low protection. Some phytotoxic effect (necrosis at the cut edges of leaf discs) was observed upon treatment with highest concentrations of BTH and JA. Therefore, care was taken to use inducer concentrations below toxic levels because necrosis could interfere with biotrophic growth of *Plasmopara*. In comparison to the seedling assays (Fig. 2B), the expression of marker genes in leaf discs during BABA-IR against *P. viticola* infection was also analysed. *PR-1* was highly expressed in both water and BABA-treated leaf discs whereas *PR-4* and *LOX-9* showed potentiated expression patterns in BABA-treated leaf discs upon infection with *P. viticola* (Fig. 4B). The potentiated expression of the JA pathway was observed in BABA-treated leaf discs with a peak at 7 hours post inoculation which was similar to the seedling assay.

The role of callose in resistance against *P. viticola*.

The callose synthesis inhibitor DDG was also used to assess the importance of callose in both genetic and BABA-induced resistance. Leaf discs were treated, inoculated with *P. viticola* and the number of sporangia per leaf disc were counted 6 days after inoculation as described in materials and methods. In the case of the susceptible variety Chasselas, the number of sporangia was highest in non-treated control plants as well as in DDG-treated plants (Fig. 5A), whereas the lowest number of sporangia was observed in BABA-treated plants (Fig. 5A). The

number of sporangia increased with increasing concentrations of DDG in BABA-treated plants (data not shown). To investigate the relationship between callose deposition and sporulation, callose deposition was quantified in duplicate experiments with the same treatments. The highest callose deposition, measured as intensity of yellow in micrographs, was observed in BABA-treated Chasselas (Fig. 5B). The observed callose intensity decreased with the co-application of DDG (Fig. 5B). The variety Solaris displays genetically determined resistance against *P. viticola*, however, the number of sporangia increased significantly in DDG-treated infected Solaris plants (Fig. 5C). The number of sporangia was reversely correlated with callose deposition indicating the involvement of callose in resistance against *P. viticola* (Fig. 5D).

Since the plant hormone ABA has recently been shown to play a role in BABA-regulated callose deposition in Arabidopsis (Ton and Mauch-Mani, 2004) the potential role of ABA in defence of grapevine was investigated. Leaf discs were treated, inoculated with *P. viticola* and the number of sporangia per leaf disc were counted at 6 dpi. There was no significant level of resistance in ABA-treated plants at lower concentrations, whereas a small reduction in the number of sporangia was observed with high concentration of ABA (Fig. 5E). Minor phytotoxic effects were observed in leaf veins (brownish colour) upon the treatment with higher concentration of ABA. Interestingly, the level of resistance against *P. viticola* in ABA-treated plants was significantly lower than in JA-treated plants (Fig. 5E). To investigate the relationship between callose intensity and formation of sporangia in JA-induced resistance, callose deposition was quantified in the same experiment. The highest callose intensity was observed in JA-treated Chasselas and it decreased with the co-application of DDG. Callose intensity was much lower in ABA-treated plants than JA-treated plants (Fig. 5F).

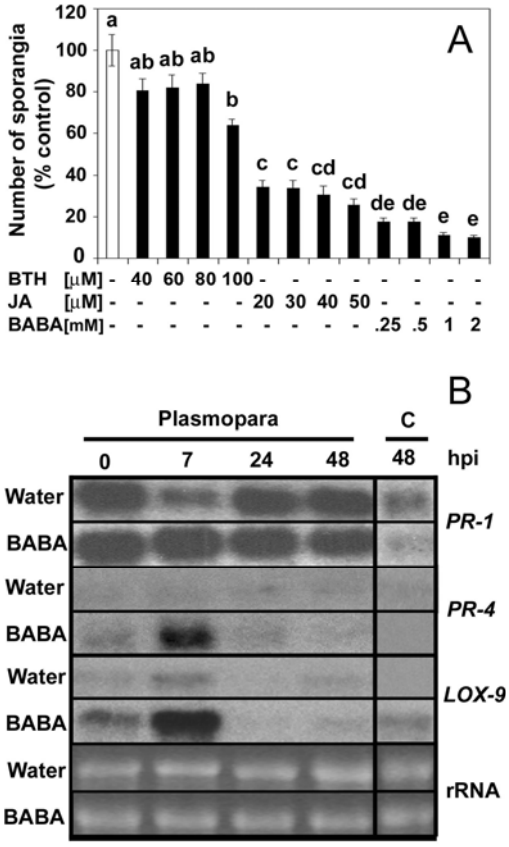

Fig. 4. Effect of BTH, JA and BABA on the infection of *Plasmopara viticola* in grapevine (Chasselas) and expression pattern of marker genes in leaf discs after infection. **A,** Leaf discs (16 discs/treatment; 4 discs/replication; 4 replications/ treatment) were punched from the third and fourth leaves from the top of a plant. They were treated with water (white bar), and BTH, JA and BABA (dark bars) 24 hours prior to challenge inoculation with sporangia of *P. viticola* ($5x10^4$ sporangia mL^{-1}). The number of sporangia (*P. viticola*) per leaf disc was determined 6 days after inoculation. Values presented are means ± sem. Different letters indicate statistically significant differences (Tamhane's test ; $\alpha = 0.05$). The experiment was repeated 3 times with similar results. **B,** Leaf discs were treated with water or BABA (1 mM) 24 hours prior to challenge inoculation with sporangia of *P. viticola* ($5x10^4$ sporangia mL^{-1}). Inoculated discs were collected at different time points as indicated. Each time point represents 16 leaf discs. RNA blots were hybridised with *PR-1*, *PR-4* and *LOX-9* probes. Ethidium bromide staining of the RNA gel (rRNA) was used to show equal loading. The experiment was repeated twice with similar results. hpi: hours post inoculation.

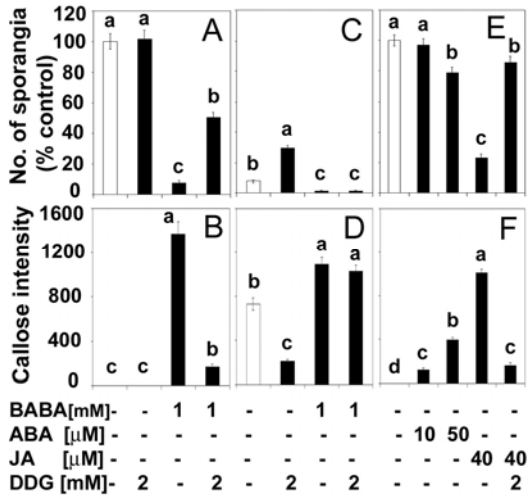

Fig. 5. Effect of different inducers and the callose synthesis inhibitor DDG on the infection of grapevine by *Plasmopara viticola* and on the intensity of callose deposition in grapevine (Chasselas). Leaf discs (16 discs/ treatment ; 4 discs/replication ; 4 replications/treatment) were punched from the third and fourth leaves from the top of a plant. Leaf discs were treated with water (white bars), BABA (**A, B, C, D**), ABA or JA (**E, F**) and/or DDG 24 hours prior to challenge inoculation with sporangia of *P. viticola* ($5x10^4$ sporangia mL^{-1}) as indicated below the figure.

A and **E,** Number of sporangia (*P. viticola*) per leaf disc (Chasselas) 6 days after inoculation. **B** and **F**, Callose intensity in leaf discs of Chasselas (3 dpi). **C**, Number of sporangia (*P. viticola*) per leaf disc (Solaris) 6 days after inoculation. **D**, Callose intensity in leaf discs of Solaris (3 dpi). Callose quantification was performed by determining the number of fluorescent pixels corresponding to callose deposition on digital photographs of the infected leaf area. Values presented are means ± sem. Different letters indicate statistically significant differences (Tamhane's test; $\alpha = 0.05$). The experiment was repeated 3 times with similar results.

The role of the phenylpropanoid pathway in BABA-IR against *P. viticola*.

To further clarify the involvement of phenylpropanoid pathway-derived defense responses in BABA-IR of grapevine, the effect of the PAL inhibitor AIP (Zon and Amrhein, 1992) and the callose inhibitor DDG together with BABA on the infection of *P. viticola* was analyzed (Fig. 6). Leaf discs were treated, inoculated and the number of sporangia per leaf disc was determined at 6 dpi. In Chasselas, the number of sporangia was highest in non-treated control plants (Fig. 6A). There was no significant difference between non-treated control plants and plants treated either with AIP alone or with AIP together with DDG demonstrating that these treatments had no negative effect on *P. viticola*. In contrast, BABA-treated plants showed the lowest number of sporangia (Fig 6A). A small increase in the number of sporangia was observed with the co-application of AIP with BABA, and this coincided with a reduced level of lignification (data not shown). However, the number of sporangia increased significantly when DDG was applied together with AIP in BABA-treated Chasselas. In the resistant variety Solaris, AIP alone has no significant effect on the sporulation of *P. viticola* in comparison to non-treated plants (Fig. 6B). The number of sporangia increased significantly in plants treated with AIP and DDG together. BABA treated plants showed the highest protection against *P. viticola*. The strong BABA-IR in treated Solaris was not suppressed with DDG and AIP treatment (Fig. 6B).

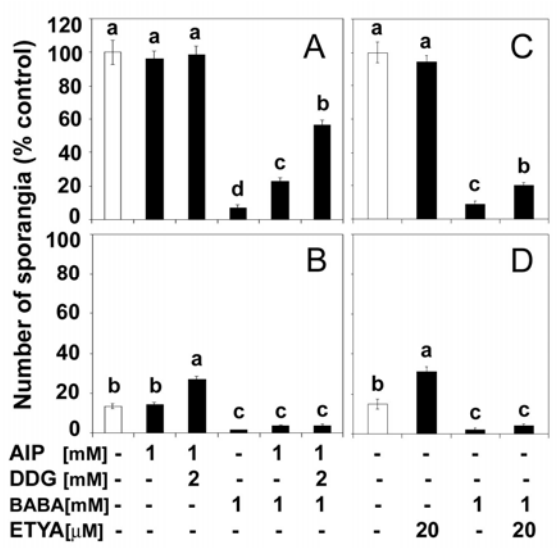

Fig. 6. Effect of the PAL inhibitor AIP, callose synthesis inhibitor DDG and the LOX inhibitor ETYA together with BABA on the infection of *Plasmopara viticola* in grapevine. Leaf discs, **A** and **C**, Chasselas; **B** and **D**, Solaris (16 discs/treatment ; 4discs/replication ; 4 replications/treatment) were punched from the third and fourth leaves from the top of a plant and were treated with water (white bars), and BABA, AIP and/or DDG (**A** and **B**) 24 hours prior to challenge inoculation with sporangia of *P. viticola* ($5x10^4$ sporangia mL^{-1}). The discs were first floated for 2 hours on ETYA solution (**C** and **D**) before BABA or water control treatment applied 24 hours prior to challenge inoculation with sporangia of *P. viticola* ($5x10^4$ sporangia mL^{-1}). Values presented are means ± sem. Different letters indicate statistically significant differences (Tamhane's test ; $\alpha = 0.05$). The experiment was repeated 3 times with similar results.

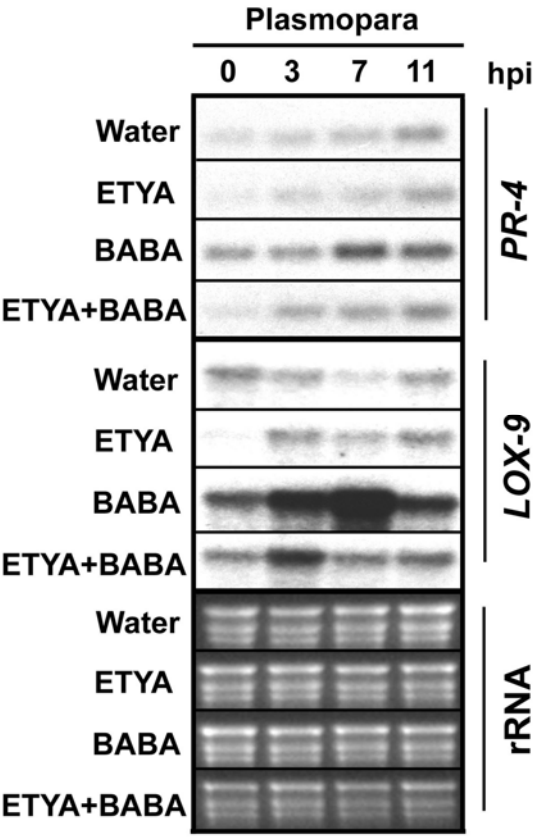

Fig. 7. Effect of the LOX inhibitor ETYA on the expression pattern of JA-regulated marker genes. Leaf discs of Chasselas were first floated for 2 hours on water (water and BABA) or ETYA solution (ETYA and ETYA + BABA) before BABA (BABA and ETYA+BABA) or water control (water and ETYA) treatment applied 24 hours prior to challenge inoculation with sporangia of *P. viticola* ($5x10^4$ sporangia mL^{-1}). Inoculated discs were collected at different time points as indicated. Each time point represents 16 leaf discs. RNA blots were hybridised with *PR-4* and *LOX-9* probes. Ethidium bromide staining of the RNA gel (rRNA) was used to show equal loading. hpi: hours post inoculation.

The role of JA-dependent defences in BABA-IR against *P. viticola*.

To identify the role of the JA-defence signalling pathway in BABA-IR, the LOX inhibitor ETYA was used. In Chasselas, the number of sporangia was as high in ETYA-treated than in non-treated plants (Fig. 6C) indicating that ETYA has no direct effect on *P. viticola*. The low number of sporangia in BABA-treated plants increased with the co-application of ETYA, but remained lower than in control plants. In Solaris, the number of sporangia increased significantly with the application of ETYA (Fig. 6D). However, ETYA was not able to suppress the strong resistance in BABA-treated Solaris. To verify the inhibitory effect of ETYA on the JA pathway in grapevine leaf discs the expression pattern of marker genes for this pathway was analyzed. Pre-treatment with ETYA prior to BABA treatment strongly reduced the expression of both *PR-4* and *LOX-9* following infection with *Plasmopara* (Fig. 7).

DISCUSSION

Our results confirm that BABA protects grapevine from infection by *P. viticola* (Cohen et al., 1999). BABA induced a significant level of resistance against *P. viticola* in grapevine seedlings (Chasselas) when applied as soil drench, as sparse sporulation was observed at 10 dpi only with the lowest concentration of BABA. This suggests that BABA translocated through the root system to protect the different plant parts and delayed the sporulation of *P. viticola*. These results confirmed earlier findings of Cohen and colleagues (1999) and showed that the interaction of Chasselas with downy mildew is amenable for further studies of BABA-IR in grapevine.

Leaf disc assays showed a big difference in the reactions of the two varieties of grapevine following infection with downy mildew. The susceptible variety (Chasselas) developed early,

large lesions (visible 3-4 days after inoculation) with clusters of sporangiophores and conspicuous sporulation, while the resistant variety (Solaris) developed necrotic spots with late, sparse sporulation. The necrosis observed in BABA-treated Chasselas plants indicates the accumulation of phenolic compounds. The single distorted sporangiophores bearing a low number of sporangia that were observed in BABA-treated Chasselas are reminescent of the occasional sparse sporulation in the (non-treated) resistant cultivar Solaris. Gindro et al. (2003) showed that in Solaris *P. viticola* cannot complete its life cycle. This is probably due to a very rapid defence response including the deposition of callose-like material in the sub-stomatal cavity that takes place as soon as germ tubes try to penetrate the stomata of this resistant cultivar.

Histochemical studies were carried out to examine the biochemical changes at the cellular level upon infection with *P. viticola*. Callose deposition and lignification at the cellular level could contribute to prevent the infection of *P. viticola* in BABA-treated plants. Flavonoids have been shown to play a key role in another resistant *Vitis* species (*Vitis rotundifolia*), and resveratrol at an early stage, and flavonoids and lignin at a later stage have been proposed to play a role in restricting the growth of *P. viticola* in intermediate resistant species (*Vitis rupestris*) (Dai et al., 1995). In plants, a pre-treatment with low concentrations of SA or BABA primes the cells to react more quickly and efficiently to subsequent elicitor treatment or pathogen attack (Conrath et al., 2002). Since the biochemical changes at the cellular level in BABA-treated grapevine plants occurred only upon infection with *P. viticola* this suggests that here too BABA might act via priming. Biochemical changes were observed in epidermal, mesophyll and guard cells, and in cell walls of BABA-treated plants after inoculation with *P. viticola*. Callose deposition and lignification became visible as early as 24 hours after inoculation and increased during the following days. In the resistant variety Solaris, callose

deposition and lignification were more pronounced in BABA-treated than in non-treated plants. Since stomata are crucial for *P. viticola* infection, callose deposition and lignification in stomatal cells might restrict penetration and development of *P. viticola* in BABA-treated plants.

Application of the callose synthesis inhibitor DDG had no direct toxic effect on *P. viticola*, but it suppressed BABA- and JA-induced resistance in Chasselas as well as basal resistance in Solaris. BABA- and JA-induced resistance positively correlated with intensity of callose deposition in Chasselas. Jakab et al. (2001) reported that the higher and faster accumulation of callose in ovules lead to the sterility of BABA-treated Arabidopsis flowers which could be reversed by a DDG treatment.

Callose deposition decreased with increasing concentrations of DDG and was inversely correlated with the number of sporangia. The highest callose intensity was observed in BABA-treated Solaris but DDG failed to completely suppress this strong resistance . BABA- and JA-induced resistance in Chasselas and basal resistance in Solaris were significantly reduced with DDG treatment but this did not result in a susceptibility comparable to the one of non-treated Chasselas plants. This indicates that other factors besides callose deposition must be involved in the resistance process. Therefore, callose deposition is a major, but not the only factor in BABA-IR against *P. viticola*.

The phenocopy HR lesions in BABA-treated grapevine infected by *P. viticola* have been shown to acumulate lignin-like deposits (Cohen et al., 1999). Callose accumulation has been observed in tolerant cultivars at later stages of infection indicating that mechanisms different from callose deposition are also involved in defence against *P. viticola* (Kortekamp et al., 1997).

To elucidate further possible mechanisms contributing to BABA-IR against *P. viticola*, we examined the involvement of SA-, JA- and ABA-dependent defences. A higher protection was observed against *P. viticola* in BABA- than in JA-treated plants and only a very limited effect could be observed with BTH and ABA. Callose accumulation was higher in BABA- and JA-treated plants than in BTH- (data not shown) and ABA-treated plants. The number of sporangia in BABA- and JA-treated plants was inversely correlated with the intensity of callose deposition.

The lack of effects of ABA is in accordance with a report by Lu et al. (2001) describing the presence of higher ABA concentrations in grapevine shoots with symptoms of Pierce's disease than in healthy shoots. In tomato plants, the susceptibility to *Botrytis cinerea* was enhanced by ABA treatment (Audenaert et al., 2002). In contrast ABA has been shown to regulate plant responses to abiotic stresses (Xiong et al., 2002) and to induce resistance against necrotrophic pathogens in Arabidopsis (Ton and Mauch-Mani, 2004). The failure of the SA-analogue BTH to induce resistance against downy mildew of grape agrees with previous findings that BABA-IR in Arabidopsis against *H. parasitica* is SA-independent (Zimmerli et al., 2000). The role of SA has been investigated in transgenic tobacco which constitutively express the salicylic acid hydroxylase gene leading to a continuous transformation of SA into catechol (NahG, Delaney, 1997). BABA was found to be equally effective in NahG-expressing as in wild type Arabidopsis against downy mildew suggesting that BABA may trigger a resistance-mediating signal transduction pathway downstream of SA (Zimmerli et al. 2000).

In grapevine, the JA pathway rather than the SA and ABA signaling pathways seems to be involved in induced resistance against downy mildew. Thomma et al. (1998) reported that the JA- and camalexin- insensitive mutants of Arabidopsis were susceptible to *Alternaria brassicicola*. In grapevine the exogenous application of JA induced resistance not only to

pathogens, but also to foliar and root herbivores (Omer et al., 2000). In our experiments, BABA- and JA-induced resistance against *P. viticola* were similarly based on high callose deposition, indicating that callose deposition might depend on the JA-defence signal pathway. In contrast, BABA-primed callose deposition in Arabidopsis is based on ABA- rather than JA-signaling (Ton et al., 2005). A recent study of these two signaling pathways in Arabidopsis shows that they might have an antagonistic role in stress responses (Anderson et al., 2004). Therefore, further studies are needed to clarify the potential interactions of JA and ABA on callose deposition during BABA-IR in different plants.

The plant hormones SA and JA play key roles in the regulation of defence responses to different pathogens and herbivores. To investigate the involvement of signal transduction pathways in BABA-IR the expression pattern of marker genes of the SA and JA pathways were tested in both seedling and leaf disc assays. In seedlings the overall expression of *PR-1* was low during downy mildew infection but an earlier and higher expression was observed following BABA-treatment. This indicates that BABA is able to prime the SA signaling pathway in grapevine. However, the failure of BTH to induce resistance as well as the constitutively high expression of *PR-1* in leaf discs show that in this case, the SA pathway does not contribute to BABA-IR. On the other hand the clear potentiated expression of *LOX-9* and *PR-4* in both BABA-treated seedlings and leaf discs following *P. viticola* infection further supports an involvement of the JA signal transduction pathway in BABA-IR. This is comparable to results that Arabidopsis mutants affected in JA biosynthesis or signalling are more susceptible to *Pythium mastophorum* (Vijayan et al., 1998) as well as to insect herbivory (McConn et al., 1997). A possible role for 9-LOX in induced resistance in grapevine has also been proposed by Aziz et al. (2003). They showed that *9-LOX* gene expression correlated with an increased defence following laminarin treatment of grapevine. Similarly, Aziz et al.

(2004) reported that oligogalacturonides stimulated *PR* gene activity which correlated with a reduced infection of grapevine plants by *Botrytis cinerea*.

Since in grapevine it is not possible to assess the role of signalling pathways by using mutants in these pathways, the involvement of different signalling compounds were tested through biochemical inhibition of the pathways of interest.

The role of lignification and of the SA-dependent signaling pathway in BABA-IR was tested by applying the PAL inhibitor AIP. The resistance in BABA-treated Chasselas decreased only poorly with co-application of AIP, and AIP also failed to suppress the basal resistance in Solaris against *P. viticola*. However, BABA-IR in Chasselas and basal resistance in Solaris decreased significantly when plants were treated with both the callose inhibitor DDG and AIP. The synergistic effect of DDG and AIP on the resistance indicates that in addition to callose deposition, other factors such as lignification might enhance the defence reaction against *P. viticola*. Mauch-Mani and Slusarenko (1996) reported that PAL is involved in the synthesis of SA and precursors of lignification in Arabidopsis, and treatment of Arabidopsis with AIP shifted the interaction with *Peronospora parasitica* towards susceptibility. The low protection of *P. viticola* with BTH, an unclear expression pattern of *PR-1* and the weak effect on resistance of AIP alone rules out any major involvement of the SA signaling pathway.

Lipoxygenase inhibitors have been successfully used to inhibit wound- or elicitor-induced responses in various plants (Staswick et al. 1991; Pena-Cortes et al. 1993; Ellard-Ivey and Douglas 1996). Rickauer et al. (1997) investigated the role of LOX and jasmonate in tobacco plants infected with *Phytophthora parasitica* var. *nicotianae* by using the LOX inhibitor (ETYA). Aziz et al. (2003) observed that laminarin did not induce cell death in grapevine, but increased defense-related gene expression (including *LOX-9*), phytoalexin (resveratrol and viniferin) production and activity of chitinases and β-1,3-glucanases. At the same time,

laminarin induced protection against *B. cinerea* and *P. viticola*. In our study, the LOX inhibitor ETYA was combined with BABA. ETYA itself had no toxic effect on *P. viticola* but could reduce the JA-dependent responses of grapevine. The BABA-IR in Chasselas and the basal resistance of Solaris decreased with co-application of BABA and ETYA. The partial protection against *P. viticola* by JA, the potentiated expression of marker genes of the JA pathway and the observed suppression of resistance by ETYA strengthen the argument of an involvement of the JA signal transduction pathway in BABA-IR against *P. viticola*.

In conclusion the present study shows that BABA induced resistance against *P. viticola* in both susceptible and resistant varieties of grapevine. The resistance in BABA-treated Chasselas plants and the basal resistance in Solaris depended to a large extent on the deposition of callose, which was positively correlated with BABA- and JA-induced resistance. *LOX-9* and *PR-4* gene expression was potentiated in BABA-treated plants upon infection with *P. viticola*. BABA-IR was significantly suppressed by the LOX inhibitor (ETYA) rather than by the PAL inhibitor (AIP). BTH and ABA failed to trigger induced resistance against *P. viticola*. On the other hand, the synergistic effect of AIP with DDG strengthens the hypothesis that lignification and accumulation of phytoalexins contribute, in addition to callose deposition, to the expression of resistance. Our results suggest that primed callose deposition plays a major role in BABA-IR of grapevine and is largely dependent on the JA signal transduction pathway. On-going studies on the involvement of phytoalexins and stilbene synthases will give a clearer picture of BABA-IR in grapevine against downy mildew.

MATERIALS AND METHODS

Biological materials.

Plants were raised from cuttings. Both grape varieties (Chasselas and Solaris) and an isolate of *P. viticola* (NCCR1) were obtained from RAC, Nyon, Switzerland. Seedlings were grown from stratified seeds (Chasselas) kindly provided by Syngenta, Switzerland. The seeds were planted in vermiculite at 30°C for germination. After 10-15 days, the seedlings were transferred to potting soil (Substrat 1, Klassman-Deilmann GmbH, Germany) and kept at 25°C (16 hours light). Seedlings at the 3-5 leaf stage were used in the experiments. *P. viticola* was maintained on detached leaves of grapevine, seedlings or whole potted plants by repeated inoculations at 20°C.

Leaf disc assay.

The third and fourth leaves from the top of plants were used. Leaf discs (1 cm diameter) were punched from different plants and discs were mixed. Leaf discs were inoculated with sporangia of *P. viticola* ($5x10^4$ sporangia mL^{-1}). Inoculated leaf discs were stained with Lactophenol-trypan blue (Keogh *et al*., 1980) at different time points (1, 2, 3, 4, 5, 6 days after inoculation). The stained samples were examined with a light microscope.

Treatment with BABA, BTH, JA, DDG, AIP, ETYA and ABA.

BABA, JA, the callose synthase inhibitor DDG, the LOX inhibitor ETYA and ABA were purchased from Sigma. The PAL inhibitor AIP was a gift from N. Amrhein, ETHZ, Zürich, Switzerland and the SA analogue BTH was supplied by Syngenta, Basel, Switzerland.

The chemicals were dissolved in water (ETYA and ABA were dissolved in a few drops of ethanol prior to dissolving in water) at the indicated concentrations. Leaf discs were floated (lower surface uppermost) on the chemical solutions for 24 hours in the dark. In the case of ETYA the discs were floated for 2 hours on the solution before the next treatment. The leaf discs were washed one time in water and then transferred to Petri dishes containing 3 moist Whatman filter papers (16 discs/Petri dish). Controls consisted of discs from non-treated and/or non-inoculated plants. The leaf discs (lower surface uppermost) were inoculated with a sporangial suspension of *P. viticola* ($5x10^4$ sporangia mL^{-1}; 2x10µl drops/disc). The Petri dishes were sealed with parafilm (to maintain high relative humidity) and placed in a 20°C growth cabinet (16 hours light) for the rest of the time.

Staining for Microscopy.

Staining with lactophenol-trypan blue: To observe the structures of *P. viticola*, inoculated leaf discs were stained with Lactophenol-trypan blue according to Keogh et al. (1980). The discs were incubated for 48 hours in the staining solution. The staining solution was removed and the stained discs were destained with chloral hydrate (2.5g mL^{-1}). Destained discs were mounted in chloral hydrate solution and examined with a light microscope. Hyphae, sporangiophores and sporangia were stained blue.

Histochemical examination and quantification of callose deposition : Leaf discs was stained with decolorized aniline blue (water blue; Fluka) for callose according to Smith and McCully (1978) and counterstained with 0.002% calcofluor (fluorescent brightener; Sigma, USA) to visualize oomycete structures (Zimmerli et al, 2000). The discs were stored in 0.07M Na-phosphate buffer and examined with UV light using an epifluorescence microscope (BP 340-

380 nm, LP 425 nm). Callose deposition in response to *P. viticola* was quantified according to Ton and Mauch-Mani (2004).

Lignification: Inoculated leaf discs were boiled 10 minutes in 95% ethanol and then treated with phloroglucinol (10 g in 95 mL absolute ethanol) for 3 minutes. The discs were washed in 25% HCl (Dai et al., 1995 and Cohen et al., 1999) mounted in glycerol (75%) and examined with a light microscope.

Induced resistance bioassays.

Leaf discs (16 discs/treatment ; 4 discs/replication ; 4 replication/treatment) were treated with water (control), BABA, BTH, JA, DDG, AIP and ABA in different combinations (see corresponding figures) 24 hours prior to challenge inoculation with sporangia of *P. viticola* ($5x10^4$ sporangia/mL). The discs were placed in a 20°C growth cabinet (16 hours light). The number of sporangia per leaf disc were counted 6 days post inoculation (dpi).

Seedlings of grapevine (Chasselas) were taken at the 4-5 leaf stage and placed in trays. A Petri dish was placed at the bottom of each pot. The plants were soil drenched with BABA (0.5, 1, 2, 4 mM). Four holes were punched into the soil around the base of seedlings in each pot. Different concentrations of BABA solution were poured into the holes in every treatment. Then the plants were placed in a 20°C growth cabinet (16 hours light) for 2 days. Then the plants were inoculated by dipping the leaves into a sporangial suspension of *P. viticola* ($5x10^4$ sporangia mL^{-1}). The inoculated leaves were marked with a felt pen. Inoculated seedlings were covered with a moist polythene sheet (to ensure sufficient humidity) and placed in the same growth cabinet. The seedlings were watered by adding water to the Petri dish when needed. The seedlings were observed to assess the infection (%) and disease severity at 6 and 10 dpi. Disease intensity was visually assessed using a 1-5 scale, in which : 1 = no symptoms visible ;

2 = up to 25% ; 3 = 25-50% ; 4 = 50-75% and 5 = > 75% of the leaf area is affected 6 and 10 days after inoculation. Values presented in different graphs are means ± sem (standard error mean). Different letters indicate statistically significant differences (Tamhane's test ; α= 0.05).

RNA gel blot analysis.

Total RNA was extracted by homogenizing frozen leaf tissue in extraction buffer (2% CTAB, 2% PVP K30, 100 mM tris-HCL pH 8.0, 25 mM EDTA, 2 M NaCl, 0.5 g L^{-1} spermine; 5 mL g^{-1} leaf tissue). The homogenates were extracted twice with chloroform and the RNA was precipitated using LiCl, as described by Sambrook et al. (1989). For RNA gel blot analysis, 10 µg of total RNA were separated on a 1.2% agarose gel containing 5% formaldehyde, blotted onto Hybond^{TM}-XL membranes (Amersham place, Amersham Biosciences UK limited) by capillary transfer. The RNA gel blot was hybridized at 65° C with cDNA probes labeled with ^{32}P-dCTP by random primer labeling. Blots were exposed for 24 h on X-Omat AR film (Kodak).

The ACC accession numbers of the sequences used are the following: *PR-1*: CF074675; *PR-4*: CF074510; *LOX-9*: AY159556.

ACKNOWLEDGEMENTS

We thank RAC, Nyon, Switzerland, for supplying grapevine plants and Jurriaan Ton for inspiring scientific discussions. We also thank Nikolaus Amrhein for the kind gift of AIP, and Bettina Gsell, Syngenta, for supplying us with seeds of grapevine. This project was funded by the National Centre of Competence in Research (NCCR) Plant Survival, a research programme of the Swiss National Science Foundation.

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CHAPTER 3

Identification and characterization of marker genes for the different defence signalling pathways from grapevine

Gabor Jakab, Mollah Md. Hamiduzzaman, Laurent Barnavon, Ronald van Doorn, Brigitte Mauch-Mani and Jean-Marc Neuhaus

ABSTRACT

The expression pattern of defence-related genes in grapevine (*Vitis vinifera*) was investigated. Seedlings, detached leaves and leaf discs of Chasselas plants were exposed to biotic and abiotic stresses. *PR-1* was identified as a suitable marker gene for the salicylic acid (SA)-dependent defence pathway as well as *LOX-9, OSM-1* and *PR-4*, as markers for the jasmonic acid (JA)-dependent defence pathway in grapevine. No good marker gene for the abscisic acid (ABA) signal transduction pathway was found. Using reverse transcriptase-polymerase chain reaction (RT-PCR) analysis, the expression of *Chit3*, *Gluc1*, *PIN1* and *GST1* genes was also detected. No expression of any of these marker genes was observed in very young leaves with

tendrils or in developed healthy leaves. Unlike *PR-4* and *LOX-9*, *PR-1* and *OSM-1* expression was observed in leaves with aging. An increase in the expression of *PR-4* and *OSM-1* genes was observed in seedlings upon wounding while *PR-1* expression was very high 1 day after treatment. No effect of wounding was found on the expression of the *LOX-9* gene. The *PR-1*, *PR-4* and *OSM-1* genes were highly induced in powdery mildew (*Uncinula necator*) infected leaves and their expression was associated with necrosis. In contrast, only low and temporary expression of *PR-1* and *PR-4* was observed in seedlings following infection with downy mildew (*Plasmopara viticola*). In leaf disc experiments, RT-PCR results additionally showed a temporary induction of *Chit4*, *Gluc1*, *GST1* and *HSR1* genes at 7 hours post infection, the starting time of penetration of *P. viticola* into stomata. Our results suggest that older or necrotic leaves highly express the SA-regulated *PR-1* and *OSM-1* defence-related genes in grapevine. Leaves infected with powdery mildew show a constant increase in the expression of marker genes regulated by both pathways while downy mildew only temporally induces them.

INTRODUCTION

To defend themselves against pathogens plants have evolved several strategies, such as constitutively expressed barriers and inducible defense reactions. The success of a defense mechanism depends highly on the speed and the intensity by which it is activated. If the plant fails to respond early the appropriate defenses are activated too late, the pathogen can start colonizing the plant tissue. In this situation, the plant responds by activating defenses around the sites of pathogen invasion. This so-called basal resistance contributes to slow down the colonization by the pathogen, but is too weak and too late to prevent disease. However, plants

are capable of enhancing their level of basal resistance upon appropriate stimulation. This phenomenon is known as induced resistance, and can be triggered by prior infection with an avirulent pathogen (Sticher et al., 1997), non-pathogenic rhizobacteria (Pieterse et al., 1998), or various chemical agents, such as salicylic acid (Mauch-Mani and Métraux, 1998) and *ß*-aminobutyric acid (BABA; Zimmerli et al., 2000).

The classic example of induced resistance is often referred to as systemic acquired resistance (SAR), and occurs in distal plant parts upon localised infection by a necrosis-inducing pathogen. The signaling pathway controlling SAR requires endogenous accumulation of the stress hormone salicylic acid (SA; Gaffney et al., 1993; Nawrath and Metraux, 1999), and an intact defense regulatory protein NPR1/NIM1 (Cao et al., 1994, Delaney et al., 1995). The expression of SAR, triggered either by pathogen infection or by treatment with SA or its functional analogues 2,6-dichloroisonicotinic acid (INA) or benzothiodiazole (BTH), is able to induce systemic resistance in a variety of plants against a wide range of microbial pathogens (Oostendorp et al., 2001) and is tightly associated with the transcriptional activation of genes encoding pathogenesis-related proteins (PRs; Van Loon,1997). The expression pattern of PR genes is therefore often used as a marker for induction of local and systemic plant defenses.

Besides pathogen-induced SAR, some selected strains of non-pathogenic rhizobacteria can also induce a distinct systemic resistance named rhizobacteria-mediated induced systemic resistance (ISR) (Pieterse et al., 1996; Van Loon et al., 1998 Iavicoli et al., 2003). In Arabidopsis, the induction of ISR by different root-colonizing bacteria has been reported to function independently of SA (Pieterse et al., 1996; Iavicoli et al., 2003), indicating that rhizobacteria-mediated ISR is controlled by a different signaling pathway than pathogen-induced SAR. ISR triggered by the *Pseudomonas fluorescens* strain WCS417r functions independently of SA, but requires responsiveness to the plant hormones jasmonate (JA) and

ethylene (ET). Although the expression of ISR is not associated with transcriptional activation of SA-inducible *PR* genes (Pieterse et al., 1996; Van Wees et al., 1997), it also requires an intact NPR1/NIM1 protein. Hence, the NPR1/NIM1 protein functions in the signaling pathway of both SAR and ISR, illustrating its multifunctional role in the defense signaling network of the plant.

JA and ET play important roles as signal molecules mediating disease resistance in response to necrotrophic fungal pathogens (Penninckx et al., 1996, Reymond and Farmer, 1998). Recently, Farmer and colleagues (2003) further reported that JA as well as oxophytodienoic acid (OPDA) can activate and repress expression of different sets of genes in plants.

Reducing the biosynthesis of, or sensitivity to either JA or ethylene can also render plants more susceptible to pathogens and insects. For example, ethylene-insensitive tobacco plants lost their ability to protect the soil-borne oomycete *Pythium sylvaticum* (Knoester et al., 1998). Similarly, Arabidopsis mutants affected in JA biosynthesis or signalling are more susceptible to *Pythium irregulare* (Staswick et al., 1998) and insect herbivory (McConn et al., 1997; Stout et al., 1999). Most of the JA/ET-dependent defense responses analyzed to date are SA-independent. For instance, the plant defensin gene *PDF1.2* is induced concomitantly by the JA and ET signalling pathways but not by SA. However, its induction is enhanced in transgenic *NahG* plants (Penninckx et al., 1998) indicating that SA may down-regulate the JA/ET signalling. In *eds1* and *pad4* mutant plants with reduced SA levels, elevated JA-dependent gene expression in response to inducers was also observed, providing genetic evidence for the interference of SA with JA-dependent signalling (Gupta et al., 2000). Very recently, Anderson and colleagues (2004) reported that the antagonistic interactions between multiple components of ABA and the JA-ethylene signalling pathways modulate gene expression in response to biotic and abiotic stresses in Arabidopsis.

The phytohormone abscisic acid (ABA) is involved in the regulation of many physiological stresses in plant. ABA is best known to regulate the plant responses during abiotic stress, like drought, cold and salinity (Zhu, 2002). Recent publication shows, that ABA can also be involved in defence against biotic stress. Exogenous application of ABA prior to inoculation induces resistance against necrotrophic pathogens in Arabidopsis (Ton and Mauch-Mani, 2004) as well as regulates plant responses to abiotic stresses (Xiong et al., 2002). In contrast, the susceptibility to *Botrytis cinerea* in tomato plants was enhanced by an ABA treatment (Audenaert et al., 2002) and a higher ABA concentration was observed in shoots of grapevine with Pierce's disease symptoms than in healthy shoots (Lu et al., 2001). To study defense reactions in plants aging has been studied, however little is known about the molecular mechanisms of age-related resistance (ARR). Kus and colleagues (2002) reported that the ability to accumulate SA is necessary for the ARR-response in Arabidopsis against *P. syringae* pv. *tomato*.

All these plant hormones are involved in a local and systemic induction of various PR proteins responding to the different biotic stresses (Van Loon and Van Strien, 1999). Some PR proteins may be effective in inhibiting pathogen growth, multiplication and/or spread, while others may be responsible for maintenance of SAR (Ryals et al., 1996). Most PR proteins belong to gene families and exist in both basic and acidic isoforms (Brederode et al., 1991). Acidic PR proteins are predominantly extracellular and their expression is characteristically induced after the accumulation of SA (Malamy et al., 1990; Métraux et al., 1990). In contrast to their acidic counterparts, many basic PR proteins are vacuolar and do not accumulate in response to SA or during the establishment of SAR. The expression of basic PR proteins can be triggered by ET (Brederode et al., 1991; Eyal et al., 1993) and/or by JA (Penninckx et al., 1996; Reymond and

Farmer, 1998) and is under the control of an organ-specific expression program (Eyal et al., 1993; Thomma and Broekaert, 1998).

Until now no marker genes for the SA, JA and ABA signal transduction pathways are characterized in grapevine. To be able to further study the involvement of these defense pathways in BABA-induced resistance (BABA-IR) of grapevine, a total of 27 putative defense-related genes were tested for SA- , JA- and ABA-inducibility in detached leaves as well as in seedlings. The expression patterns of marker genes were tested in leaves of different ages aging as well as with downy or powdery mildew symptoms. Marker genes for different defence signalling pathways were thus identified in grapevine.

MATERIALS AND METHODS

Biological materials

Grapevine plants (variety Chasselas) were raised from cuttings, obtained from Agroscope RAC, Changins, Switzerland. For seedlings, the seeds (Chasselas) (stratified at Syngenta, Switzerland) were sown on soil (Substrat 1, Klassman-Deilmann.GmbH, Germany) at 30°C for germination. After 10-15 days, the germinated seedlings were transfer to potted soil (22-25°C). Seedlings at the stage of 3-5 leaves were used in the experiments. Downy mildew experiments were carried out using an isolate of *Plasmopara viticola* collected from Agroscope RAC, Changins, Switzerland. *P. viticola* was maintained on detached leaves of grapevine, seedlings or whole potted plants by repeated inoculations at 20°C.

For cDNA isolation grapevine leaves (*Vitis vinifera* L., cv. Chasselas) were harvested in Neuchâtel vineyards (Domaine C. Rossel and Domaine A. Perret) before harvest. Leaves were

immediately frozen in liquid nitrogen and stored at –80 °C until use. Leaves were powdered with pestle and mortar and further used for total RNA extraction.

Chemicals

DL-β-aminobutyric acid (BABA), jasmonic acid (JA) and abscisic acid (ABA) were purchased from Sigma, the SA analogue BTH (Benzothiadiazole) was given by Syngenta. The chemicals were dissolved in water (ABA was first dissolved in a few drops of ethanol prior to dilution in water) at the indicated concentrations.

Bioassays for the different stress treatments

The third and fourth leaves from the top of plants were used. Leaf discs (1 cm diameter) were punched from different plants and were mixed. Leaf discs (lower surface up) were floated on water (control), on the solutions of BTH (0.5 mM), JA (0.1 mM) or ABA (0.1 mM) for 24 hours in the dark. The leaf discs were washed once in water and then transferred to Petri dishes containing 3 moist Whatman filter papers (16 discs/Petri dish). Controls consisted of discs from non-treated and/or non-inoculated plants. The petioles of detached leaves were dipped into the solutions for one or two days prior to harvest. The leaf discs were inoculated with sporangia of *P. viticola* ($5x10^4$ sporangia mL^{-1}) and placed in a 20°C growth cabinet (16 hours light). Pots with seedlings were placed in trays, with a Petri dish at the bottom of each pot. The pots were drenched using four holes/pot into the soil around the base of seedlings. The same solutions as for leaf discs were poured into the holes in every treatment. Plants were inoculated by dipping the leaves into a suspension of *P. viticola* ($5x10^4$ sporangia mL^{-1}). Inoculated leaves were marked with a felt pen. Inoculated seedlings were covered with a moist polythene sheet (to ensure sufficient humidity) and placed in the same growth cabinet. The

seedlings were watered by adding water to the Petri dish when needed. For the wounding experiments, the seedlings were soil drenched with water or BABA (0.5 mM) for 2 days prior harvest. The leaves were wounded by forceps at different time points. Leaves of the cutting-plants infected naturally by powdery mildew were collected in different stages of symptoms. The leaf discs, detached leaves or seedlings were harvested at different times, frozen in liquid N_2 and stored at –80°C. The experiments were repeated twice with similar results.

RNA extraction and gel blot analysis

Total RNA was extracted by homogenizing frozen leaf tissue in extraction buffer (2% CTAB, 2% PVP K30, 100 mM Tris-HCL pH 8.0, 25 mM EDTA, 2 M NaCl, 0.5 g L^{-1} spermine; 5 mL g^{-1} leaf tissue). The homogenates were extracted with phenol and chloroform and the RNA was precipitated using LiCl, as described by Sambrook *et al*. (1989). For RNA gel blot analysis, 10 µg of total RNA were separated on a 1.2% agarose gel containing 5% formaldehyde, blotted onto Hybond^{TM}-XL membranes (Amersham, Amersham Biosciences UK limited) by capillary transfer. The RNA blot was hybridized at 65° C with cDNA probes labeled with α-^{32}P-dCTP by random primer labeling kit. Blots were exposed for 24 h on X-Omat AR film (Kodak).

Synthesis of partial cDNA clones

1 µg total RNA was used as template for reverse transcription (RT) reactions according to the supplier's instructions (ImPromII™ Reverse Transcription System, Promega). Degenerate PCR primers corresponding to known protein families involved in plant resistance were designed from consensus regions in sequence alignments. PCR products were separated by

electrophoresis in a 1.2 % agarose gel and purified using a gel extraction kit (JETsorb, Genomed).

Reverse transcriptase-polymerase chain reaction (RT-PCR)

The isolated total RNA from detached grapevine leaves or leaf discs was converted into first-stranded cDNA using the Omniscript Reverse Transcription Kit (Qiagen, Basel, Switzerland). The cDNA was used as template for PCR with 20 - 25 cycles at 54 °C annealing temperature with primers for the genes of interest. Actin was used as constitutive marker gene to compare the concentration of cDNA from different samples. The amount of RT-PCR product for agarose gel loading was calculated for each sample using the actin RT-PCR product as standard. Subsequently, RT-PCR products were electrophoretically separated in 1x TAE buffer on a 1.0% agarose gel. Intensity of the PCR bands was analysed using the Bio Rad Multi-Analyst version 1.1.

Cloning gene-specific PCR fragments

The PCR products of genes of interest were ligated into the pGEM®-T Easy vector using a cloning kit (Promega, USA). Ligation mixtures were incubated overnight at 4 °C to obtain the maximum number of transformants. Ligation products were transformed into competent *E. coli* bacteria cells (XL1-blue) using a 90 seconds heat-shock at 42 °C. The bacterial cells were spread onto LB agar plates containing ampicilin/IPTG/X-Gal. Plasmid minipreps were performed by the alkaline lysis method (Sambrook and Russell, 2001). Incorporation of the PCR fragment into the vector was checked by a PCR reaction using the M13 and M13rev primers.

Sequence analysis

DNA was sequenced with the Labstation Thermo Sequenase labelled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Pharmacia Biotech, Inc) using M13 and M13 Reverse primers, and were analysed with a DNA Sequencer (Model 4000L, MWG-BIOTECH). Sequence analysis was performed by similarity search using the BLASTX+BEAUTY Web site.

Following defense-related sequences were obtained: pathogenesis-related 1 (*PR-1*; Acc.No.: CF074675); *PR-4* (CF074510), osmotin (*OSM-1*; Y10992), lipoxygenase (*LOX-9;* AY159556), chitinases (*Chit3*, Z68123; *Chit4*, AY137377), ß-1,3-glucanase (*Gluc1*, AF239617), serine proteinase inhibitor (*PIN1*, AY156047), phenylalanine ammonia lyase (*Pal1*, Sparvoli et al., 1994), glutathion-S-transferase (*GST1*, AY156048), callose synthase (*CS,* AJ430780), stilbene synthase (*STS1*, AB066275) and hypersensitive response 1 (*HSR1*, AF487826).

RESULTS

Identification of marker genes for the different defence signalling pathways from grapevine

In order to elucidate the molecular mechanisms behind BABA-IR in grapevine, marker genes for the SA- and JA-dependent defence response were needed. As only few grapevine sequences related to defence responses were available, we developed a PCR-based strategy using degenerate primers for conserved regions of defence genes. This strategy led to the isolation of 261 clones which were submitted to GenBank (NCBI) for the IGGP (International

Grape Genome Project) under the accession numbers CF589266 to CF589278 and CF074508 to CF074755.

Analysis of the sequences revealed cDNAs coding for defence genes like aminotransferases, chitinases, *PR-1*, *PR-4*, *PR-10*, thaumatin-like proteins, and stilbene synthases. The expression pattern of 27 putative defense-related genes was tested in detached grapevine leaves and in seedlings (Chasselas) upon exogenous application of BTH, JA or ABA, using macro-array analysis, RNA blot analysis (Northern), or RT-PCR. In Northern blot analysis, *PR-1* was highly expressed in both BTH-treated detached leaves (Fig. 1A) and seedlings (Fig. 1B) and increased in the days following the treatment (dpt). The expression of *PR-4*, *OSM-1* and *LOX-9* was very high in JA-treated seedlings with peaks at the first day of treatment (Fig. 1B). *LOX-9* expression was also high in JA-treated detached leaves, with a peak at 2 dpt, while the expression of *PR-4* and *OSM-1* was similar in both JA- and BTH-treated detached leaves (Fig. 1A). These genes were only expressed at low levels in either ABA-treated seedlings or detached leaves.

To investigate whether the observed discrepancies between detached leaves and seedlings in the expression pattern particularly of the JA-regulated genes were due to wounding during leaf detachment, wounding experiments were performed with grapevine seedlings. The leaves were wounded and RNA was extracted from the treated plants at 1 and 2 dpt. Indeed, both the SA-regulated *PR-1* gene and the JA-regulated *PR-4* and *OSM-1* genes were highly expressed at 24 hours after wounding, but expression of *LOX-9* was not observed (Fig. 2).

Therefore RT-PCR was used in detached leaves to determine the expression pattern of some more genes which could not be detected by northern blot analysis (Fig. 3). The expression of *Chit3*, *Gluc1* and *PIN1,* but not of *Pal1* was high in BTH-treated leaves with peak at 2 dpt (Fig. 3A). *Pal1* expression was a little higher in water-treated leaves at 2 dpt than in other

treatments. Expression of *OSM-1* was very high in JA-treated detached leaves with peak at 2 dpt (Fig. 3B). In contrast, expression of *LOX-9* was high in both JA- and BTH-treated leaves whereas *GST1* was higher in BTH-treated leaves than in ABA- and water-treated leaves. The expression of several genes was lower in ABA-treated than in BTH-, and JA-treated leaves (Fig. 3). *PIN1* expression at 2 dpt was lower in ABA-treated leaves than in BTH-treated, but was higher than in JA-treated leaves (Fig. 3A).

This analysis revealed that *PR-1* is a suitable marker for the SA-dependent defence pathway whereas *LOX-9, OSM-1* and *PR-4* genes are appropriate markers for activation of the JA-dependent defence response in grapevine. No good marker gene for ABA signalling was found.

Expression pattern of marker genes in grapevine leaves during aging

To elucidate the role of age on defence reaction in grapevine, healthy leaves were collected from potted plants: very young leaves (at top) including tendrils, 4-5th mature leaves from the top and older leaves (10-12th from top). The expression of *PR-1*, *PR-4*, *OSM-1* and *LOX-9* genes was not observed in very young leaves nor in 4-5th mature leaves (Fig. 4). In older leaves, the expression of *PR-1* was very high and *OSM-1* was also detected, although to a lower extent, neither *PR-4* nor *LOX-9* expression increased during aging.

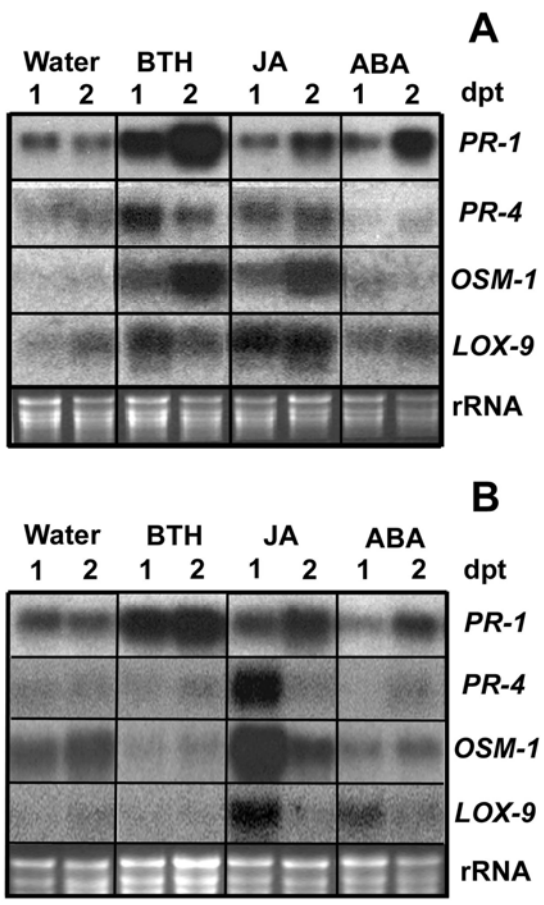

Fig. 1. A. Expression pattern of *PR-1*, *PR-4*, *OSM-1* and *LOX-9* genes in detached leaves (third and fourth leaves from the top). The petioles were put into the solutions for one or two days. Leaves were treated with water, BTH (0.5 mM), JA (0.1 mM) or ABA (0.1 mM), and total RNA was extracted at one and two days after treatment. Each time-point represents 1 g of leaf materials (8 leaves from 4 different plants). RNA blots were hybridised with *PR-1*, *PR-4*, *OSM-1* and *LOX-9* probes. Ethidium bromide staining of the RNA gel (rRNA) was used to show equal loading. dpt: days post treatment. B. Expression pattern of the *PR-1*, *PR-4*, *OSM-1* and *LOX-9* genes in seedlings of grapevine (Chasselas) upon treatments. The seedlings were soil-drenched with water, BTH (0.5 mM), JA (0.1 mM) or ABA (0.1 mM) and total RNA was extracted at different time points after treatment. Each time-point represents 1 g of leaf materials (8 leaves from 4 different plants). RNA blots were hybridised with *PR-1*, *PR-4*, *OSM-1* and *LOX-9* probes. Ethidium bromide staining of the RNA gel (rRNA) was used to show equal loading. dpt: days post treatment.

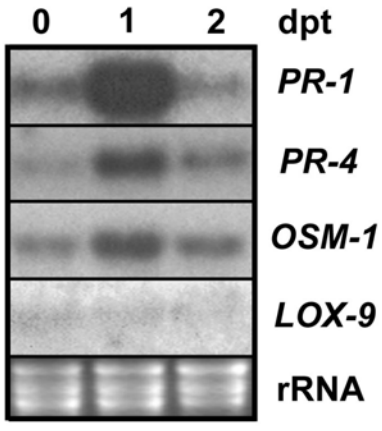

Fig. 2. Expression pattern of the *PR-1*, *PR-4*, *OSM-1* and *LOX-9* genes in seedlings of grapevine (Chasselas) upon wounding. The leaves of grapevine seedlings were wounded by forceps. Total RNA was extracted at different time points after wounding. Each time-point represents 1 g of leaf materials (8 leaves from 4 different plants). RNA blots were hybridised with *PR-1*, *PR-4*, *OSM-1* and *LOX-9* probes. Ethidium bromide staining of the RNA gel (rRNA) was used to show equal loading. dpt: days post treatment.

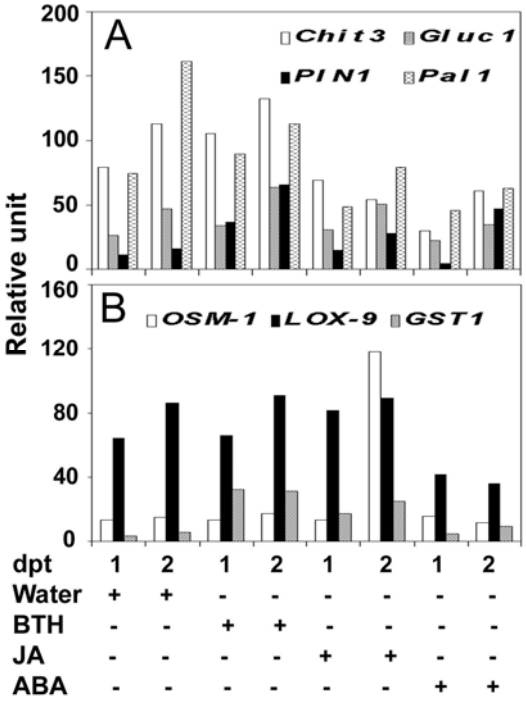

Fig. 3. RT-PCR analysis of gene expression in detached grapevine leaves (Chasselas) treated with water, 0.5 mM BTH, 0.1 mM JA or 0.1 mM ABA. Expression pattern of *Chit3*, *Gluc1*, *PIN1* and *Pal1* (A); *OSM-1*, *LOX-9* and *GST1* (B) genes. Leaves were harvested and total RNA was extracted at one and two days after starting the different treatments. Each time-point represents 1 g of leaf materials (8 leaves from 4 different plants). Intensity of the RT-PCR signal was standardized for all treatments and time points using actin as the marker for measure of the cDNA level. Relative unit is the change of expression level of a given time point and treatment compared to the lowest expression level of the given gene in this experiment (using Bio Rad Multi-Analyst version 1.1). dpt: days post treatment.

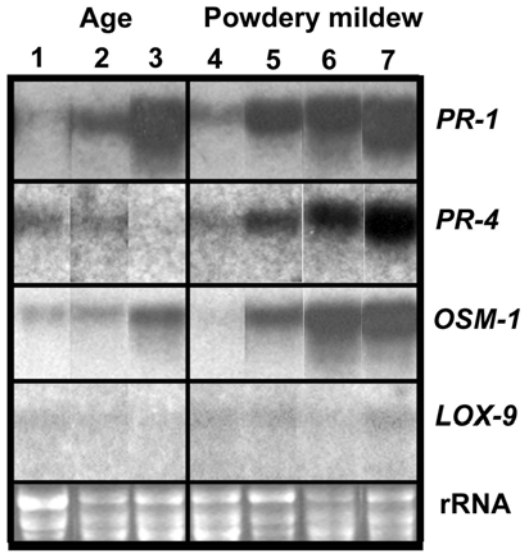

Fig. 4. Expression pattern of *PR-1*, *PR-4*, *OSM-1* and *LOX-9* genes in detached leaves of grapevine (Chasselas) upon aging and powdery mildew infection. 1 to 3 are aging: 1) very young leaves at the top of plant with tendrils, 2) 4-5th leaves from the top (developed healthy leaves), 3) older leaves (8-12th leaves from top).

Different phases of powdery mildew infection: 4) leaves with very small whitish spots (early stage of necrosis), 5) leaves with yellowish necrosis (developed necrosis), 6) powdery mildew infected leaves with brownish sporulation, and 7) very strong necrosis (leaves were yellowish and started to dry).

Leaves were collected from the cutting-plants and total RNA was extracted. Each time-point represents 1 g of leaf materials (8 leaves from 4 different plants). RNA blots were hybridised with *PR-1*, *PR-4*, *OSM-1* and *LOX-9* probes. Ethidium bromide staining of the RNA gel (rRNA) was used to show equal loading.

Induction of marker genes upon infection with powdery or downy mildew

To investigate the role of powdery mildew infection on the expression pattern of defence-related marker genes, the naturally infected leaves were collected from potted plants with different symptoms. The expression of different marker genes were tested in leaves with distinct symptoms of infection with powdery mildew (*Uncinula necator*): leaves with small whitish spots, leaves with light necrosis and leaves with very strong necrosis. The *PR-1* and *OSM-1* genes were highly expressed in plants infected with powdery mildew as well as in leaves with very strong symptoms of necrosis whereas expression was low in light necrotic leaves (Fig. 4). The expression of *PR-4* was low in leaves infected with powdery mildew as well as in light necrotic leaves, but highly expressed in leaves with very strong necrosis. The expression of *LOX-9* was not observed in leaves with any type of symptoms. The expression of *PR-1*, *PR-4* and *OSM-1* was very high in leaves with strong necrosis whereas expression was not observed in healthy leaves (without any symptom) and leaves with very few light (whitish) spots.

To explore the expression of defense-related genes upon infection with downy mildew (*Plasmopara viticola*) leaf discs and seedlings were inoculated and total RNA was extracted in different time points. In seedlings, *PR-1* was expressed at 11 hpi with peak at 24 hpi when *PR-4* was peak at 17 hours earlier (Fig. 5). Expression of *OSM-1* was observed after 3 hours of inoculation with a peak at 48 hpi. Expression of *LOX-9* was not observed in seedlings following infection. In leaf discs, *PR-1* was expressed in all time points with peak at 48 hpi (data not shown). The expression of *PR-4* and *LOX-9* gene was not observed in leaf discs following infection with *P. viticola*.

RT-PCR was used to observe expression patterns in leaf discs of some more genes following infection with *P. viticola* (Fig. 6). The expression of *Chit4*, *Gluc1* and *GST1* (Fig. 6A), *OSM-*

1, *LOX-9*, *PIN1* and *HSR1* (Fig. 6B) was high at 7 hpi. *Chit4* (Fig. 6A), *PIN1* and *HSR1* (Fig. 6B) expression was observed with peak at 7 hpi whereas *Pal1* (Fig. 6A) and *CS* (Fig. 6B) remained constant at all times. The expression of *PIN1* was high 7 hpi and remained high up to 48 hpi (Fig. 6B). Interestingly, induction of most of the genes except *Pal1* and *CS* was high at 7 hpi, the time of penetration of *P. viticola* into the stomata.

DISCUSSION

We have isolated 261 clones from grapevine and based on their sequence homology, 27 of them were selected for further expression analyses. The aim of this work was to identify reliable marker genes of the different signalling pathways for grapevine.

Most of the studies of defense responses in grapevine were concentrated on gene expression and activities of chitinases and glucanases (Busam et al., 1997; Robinson et al., 1997; Robert et al., 2002). Differential expression of chitinases was a reliable indicator of the SAR response in grapevine against *P. viticola* (Busam et al., 1997). The expression of genes of the phenylpropanoid pathway in grapevine was induced by SAR activators suggesting a role for CCoAOMT (S-adenosyl-L-methionine:trans-caffeoyl-coenzyme A 3-O-methyltransferase) and stilbene synthase in the disease-resistance reaction (Busam et al., 1997).

The detection of the chitinase and glucanase genes by Northern blot was not possible in our hand. Therefore we tested the expression of a clone showing high homology to PR-1 from other plants. The expression of this gene was easily detected after different stress treatment and showed high inducibility by SA, therefore it was selected as a good marker of the SA pathway. The lack of signalling mutants in grapevine prevented further proof of this hypothesis.

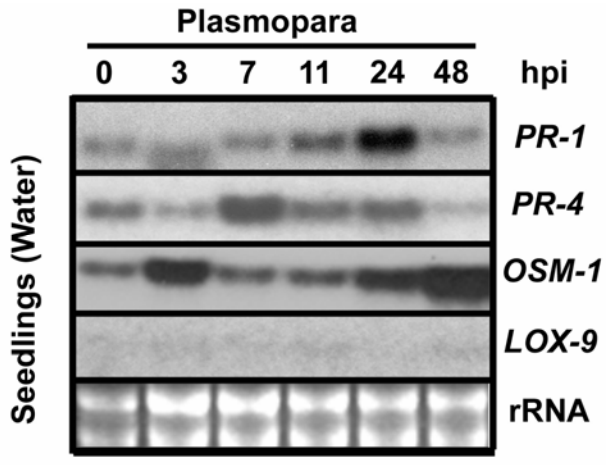

Fig. 5. Expression pattern of the *PR-1*, *PR-4*, *OSM-1* and *LOX-9* genes in seedlings of grapevine (Chasselas) upon infection with *Plasmopara viticola*. The seedlings were soil-drenched with water 2 days prior to challenge inoculation with sporangia of *P. viticola* ($5x10^4$ sporangia mL^{-1}). Total RNA was extracted at different time points after infection. Each time-point represents 1 g of leaf materials (8 leaves from 4 different plants). RNA blots were hybridised with *PR-1*, *PR-4*, *OSM-1*, *LOX-9* and *STS-1* probes. Ethidium bromide staining of the RNA gel (rRNA) was used to show equal loading. hpi: hours post inoculation.

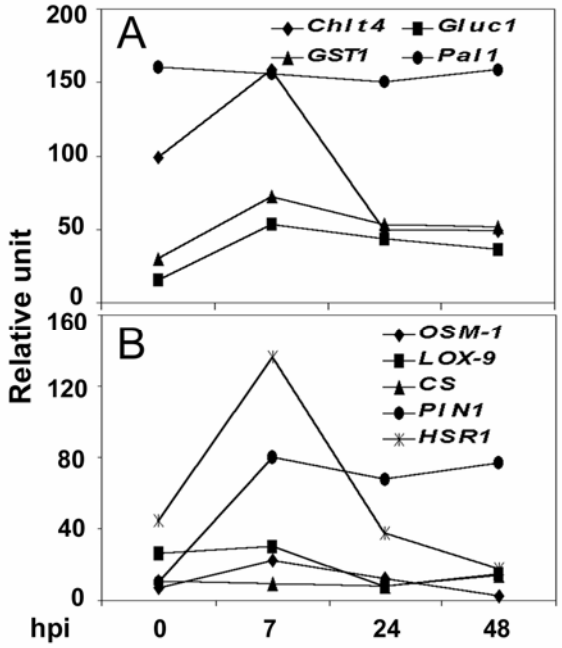

Fig. 6. RT-PCR of gene expression in water-treated leaf discs of grapevine (Chasselas) upon infection with *Plasmopara viticola*. Expression of *Chit4*, *Gluc1*, *GST1* and *Pal1* (A); *OSM-1*, *LOX-9*, *CS*, *PIN1* and *HSR1* (B) genes. Leaf discs (1 cm diameter) were punched from the third and fourth leaves from the top of a plant. Leaf discs were then treated with water 24 hours prior to challenge inoculation with sporangia of *P. viticola* ($5x10^4$ sporangia mL^{-1}). Total RNA was extracted at different time points after infection. Each time-point represents 1 g of leaf materials (20 leaf discs). Intensity of the RT-PCR signal was standardized for all treatments and time points using actin as internal control of cDNA level. Relative unit is the change of expression level of a given time point and treatment compared to the lowest expression level of the given gene in this experiment (using Bio Rad Multi-Analyst version 1.1). hpi: hours post inoculation.

The production of phytoalexins in grapevine during different defence reactions was also investigated (Pryce and Langcake, 1977; Adrian et al., 1997; Bais et al., 2000). Recent reports show that an endopolygalacturonase of *B. cinerea* (Poinssot et al., 2003) and laminarin (Aziz et al., 2003) induce other defence responses in grapevine. However, the signal transduction pathways leading to defence responses and subsequent protection against pathogens in grapevine remain unclear. Therefore we tested in parallel published defence genes and own clones during defence reactions of grapevine. We found that *PR-4, OSM-1* and *LOX-9* are good markers of the JA signalling, although the detection of LOX-9 was difficult by Northern during normal stress conditions.

In detached leaves and leaf disc assays however these genes showed an altered expression pattern indicating that an additional factor can influence their expression. We found that this factor is wounding. Wounding induces different types of defence-related genes in tomato (Chen et al., 2004), wheat (Igawa et al., 2004) and hybrid poplar (Christopher et al., 2004). In grapevine, wounding did not elicit active oxygen species (AOS) production (Papadakis and Roubelakis-Angelakis, 1999), but produced stress metabolites like resveratrol (Becker et al., 2003). To date, the wounding effect on defence responses in grapevine remained unclear. When preparing leaf discs we induced a wound response.

In conclusion, we could identify good marker genes, *PR-1* for SA signalling, and *PR-4* for JA defence reactions in grapevine. Analysis of the promoter regions of these genes could therefore supply further information on the transcriptional regulation of grapevine genes during stress responses.

Acknowledgements

We thank Roger Pezet and Hannes Richter for grapevine plants (Agroscope RAC, Changins), Jurriaan Ton for scientific suggestions and Bettina Gsell for grapevine seeds (Syngenta). This project was funded by the National Centre of Competence in Research (NCCR) 'Plant Survival' of the Swiss National Science Foundation.

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CHAPTER 4

Involvement of stilbenes in ß-aminobutyric acid-induced resistance in grapevine against downy mildew (*Plasmopara viticola*)

Mollah Md. Hamiduzzaman, Julien Bernard Jean-Denis, Gabor Jakab, Raphael Tabacchi, Jean-Marc Neuhaus and Brigitte Mauch-Mani

Manuscript in preparation

ABSTRACT

ß-aminobutyric acid (BABA), a non-protein amino acid was used to induce resistance in grapevine (*Vitis vinifera*) against downy mildew (*Plasmopara viticola*). BABA-induced resistance (BABA-IR) was observed in the susceptible variety Chasselas as well as in the resistant variety Solaris. Sporulation of *P. viticola* was strongly reduced following BABA treatment. Accumulation of stilbenes in BABA-treated plants increased with time following infection with *P. viticola*. The experiments were performed with two different systems, in leaf discs and in seedlings. In leaf discs, stilbenes were quantified from tissue at the inoculation zone as well as in the surrounding tissue. At the inoculation zone induction of stilbenes was much higher in BABA-treated leaf discs than controls, while in the surrounding tissue accumulation levels of stilbenes low. In seedlings, leaf tissue was collected from water-treated

control as well as from necrotic and non-necrotic zones of BABA-treated plants. *trans*-resveratrol and *trans*-ε-viniferin were below detection level in both non-inoculated control and in BABA-treated plants. However, accumulation of *trans*-resveratrol was primed in BABA-treated plants in both necrotic and non-necrotic zones, while *trans*-ε-viniferin was only primed in necrotic zones. *trans*-δ- and ε-viniferin, and *trans*-δ-resvepterol were toxic to zoospores. The expression of a stilbene synthase gene (*STS-1*) was higher in jasmonic acid-treated than in either BTH or ABA-treated plants. Furthermore, potentiated expression of *STS-1* was observed in BABA-treated plants upon infection. Our results suggest that BABA-IR involves priming of the *STS-1* and leads to a potentiated accumulation of resveratrol and viniferins in grapevine.

INTRODUCTION

Downy mildew (*Plasmopara viticola*) causes massive damage to grapevine (*Vitis vinifera*) all over the world. The variety Chasselas is susceptible whereas Solaris shows resistance to downy mildew. Plants respond to pathogen attack by creating signaling networks based on molecules such as salicylate, jasmonate and ethylene, followed by the accumulation of PR proteins, phytoalexins and phenolic compounds (Elad, 1997; Dong, 1998; Feys and Parker, 2000). Trans-resveratrol (3,5,4'-tryhydroxystilbene) is one of the phytoalexins which accumulates in grapevine leaves following fungal infection or UV radiation (Langcake, 1981; Langcake and Price, 1977; Adrian et al., 1997). *trans*-resveratrol is fungitoxic against *Botrytis cinerea* (Adrian et al., 1998). It also enhances the resistance of grapevine to other pathogens such as *P. viticola* (Dai et al., 1995) or *Phomopsis viticola* (Hoos and Blaich, 1990). Dai *et al*. (1995) suggested that the formation of resveratrol, flavonoids and lignin restrict the growth of

P. viticola in resistant grapevine. Aziz et al. (2003) reported that the β-1,3-glucan laminarin is an efficient elicitor of defense response in grapevine cells and plants against *Botrytis cinerea* and *P. viticola*. They observed that laminarin triggered a high production of phytoalexins (resveratrol and ε-viniferin) in grapevine cells. Recently, δ-viniferin, a resveratrol dehydrodimer produced by the oxidative dimerization of *trans*-resveratrol, was identified as a major stilbene in grapevine leaves infected by *P. viticola* (Pezet et al., 2003).

During recent years, much attention has been focused on the activation of the plant's own defense system. The activation of mechanisms by which plant defends themselves against a wide range of pathogens is known as induced resistance (Van Loon, 1997). A possible plant disease control strategy is based on the induction of the plant's own defense response with some synthetic molecules. DL-ß-aminobutyric acid (BABA) is able to potentiate the expression of defense-related genes in a variety of plants against a broad spectrum of microbial pathogens. BABA was effective to activate the plants defense against foliar diseases and against soil-borne pathogens (Jakab et al., 2001; Cohen 2000). Experiments with ^{14}C-labelled BABA clearly showed that this chemical is not metabolized in tomato (Cohen and Gisi, 1994) or in Arabidopsis (Jakab et al., 2001), ruling out a BABA-metabolite acting as an antimicrobial compound in the plant. Recently, Ton and Mauch-Mani (2004) demonstrated that BABA-induced resistance against necrotrophic pathogens is most likely based on primed callose accumulation, which is regulated by an ABA-dependent defense pathway in Arabidopsis.

Methyl jasmonate (MeJA) has been shown to induce systemic resistance and to enhance local defense reaction in parsley (Kauss et al., 1992) and to regulate proteinase inhibitors in tobacco cells (Rickauer et al., 1992). Farmer and colleagues (2003) reported that JA and the related

oxophytodienoic acid (OPDA) can activate on repress expression of different sets of genes. We have shown that the JA signal transduction pathway is involved in BABA-induced resistance against *P. viticola*, which in grapevine is based on callose deposition and lignification at the cellular level (Hamiduzzaman et al., 2005).

The objective of the work reported here was to investigate the involvement of phenolics and stilbenes like *trans*-resveratrol and viniferins in the establishment of BABA-IR in grapevine against *P. viticola*. Different staining techniques were used to visualize biochemical changes at the cellular level and stilbenes were quantified by HPLC. The reactions during BABA-IR in the susceptible variety (Chasselas) were compared to naturally occuring disease resistance in the resistant cultivar (Solaris). Leaf discs as well as seedlings (Chasselas) were used in these experiments. We further explored the involvement of a stilbene synthase gene (*STS-1*) in BABA-IR against *P. viticola*.

MATERIALS AND METHODS

Biological materials

Plants were raised as described (Hamiduzzaman et al., 2005). Seedlings at the 3-5 leaf stage were used in all experiments. *P. viticola* (strain NCCR1) was maintained on detached leaves of grapevine, seedlings or whole potted plants by repeated inoculations at 20°C.

Chemicals

DL-β-aminobutyric acid (BABA), jasmonic acid (JA), abscisic acid (ABA) and acetone were purchased from Sigma (Switzerland). BTH (Benzothiadiazole) was supplied by Syngenta (Basel, Switzerland). The chemicals were dissolved in water (ABA was dissolved in a few drops of ethanol prior to dissolving in water) at the indicated concentrations. Leaf discs were

floated (lower surface up) on the chemical solutions for 24 hours in the dark at 22 °C. The leaf discs were washed once in water and then transferred to Petri dishes containing 3 moist Whatman filter papers (16 discs/Petri dish). The seedlings (Chasselas) were soil drenched with the different solutions. Controls consisted of discs or seedlings from non-treated and/or non-inoculated plants. *trans*-ε-viniferin, *trans*-δ-viniferin and *trans*-δ-resvepterol (isolated in the Laboratory of Analytical and Organic Chemistry, University of Neuchâtel, Switzerland) were dissolved in acetone (1 % solution) at the indicated concentrations to test the effect on the mobility of *P. viticola* zoospores.

Microscopic examination

a) Structures of *P. viticola* :

Mycelia, sporangiophores and sporangia in inoculated leaf discs were stained with Lactophenol-trypan blue (Keogh et al., 1980) and examined with a light microscope.

b) Autofluorescence :

Inoculated leaf discs were placed on a glass slide with a few water droplets, covered with a cover slip and examined with an epifluorescence microscope with an UV excitation filter (BP 340-380 nm, LP 425 nm). Blue fluorescence indicated the presence of resveratrol.

c) Accumulation of flavonoids :

Flavonoid accumulation was visualized by using Wilson's reagent (Dai *et al*., 1995). Consisting of citric acid:boric acid (5:5, w/w) in 100 ml absolute methanol. Leaf discs were immersed in Wilson's reagent for 15 minutes, mounted in glycerol (75%) and examined under UV light with an epifluorescence microscope (BP 340-380 nm, LP 425 nm). Flavonoids fluoresced yellow and gallic acid derivatives (GAD) fluoresced blue.

d) Callose deposition and lignification:

Callose deposition and lignification was observed by using aniline blue and phloroglucinol-HCL staining, respectively, as described (Hamiduzzaman et al., 2005).

Induced resistance bioassays

The third and fourth leaves from the top of potted plants (Chasselas and Solaris) were taken randomly. Leaf discs (1 cm diameter) were punched and discs from different plants were mixed. Leaf discs (3x4 discs per treatment) were treated with water (control) or BABA (1 mM) 24 hours prior to challenge inoculation with sporangia of *P. viticola* ($5x10^4$ sporangia/mL). The inoculated sites (two 10 µL drops of inoculum per disc) were marked with a felt pen. The discs were placed in a growth cabinet (16 hours light, 20°C). 2 small pieces (0.3 cm diameter) were collected from the inoculation zone of each leaf disc and further 2 pieces from non-inoculated areas (Fig. 1C), and processed for determination of phenolics and stilbenes according to Pezet *et al.* (2003). Sporangia per leaf disc were counted after 6 days of inoculation.

Seedlings of Chasselas grapevine were taken at the 4-5 leaf stage and placed in trays. A Petri dish was placed at the bottom of each pot. Four holes were prepared on the soil around the base of seedlings in each pot. The plants were drenched with water (control), BABA (0.5 mM), BTH (0.5 mM), JA (0.1 mM) or ABA (0.1 mM). The plants were inoculated by dipping the leaves into a sporangial suspension of *P. viticola* ($5x10^4$ sporangia mL^{-1}). The inoculated leaves were marked with a felt pen. The inoculated seedlings were covered with a moist polythene sheet (to ensure sufficient humidity), placed in the same growth cabinet and watered by adding water to the Petri dish when needed.

To test the mobility of the zoospores a suspension of *P. viticola* (10^5 sporangia mL^{-1}) was used. Different stilbene solutions were added to a zoospores suspension and the mobility of

live zoospores was quantified in an area of 0.11 mm^2 under the microscope (Leica-Leitz, magnification 600x) during a 30 second period according to Pezet *et al.* (2004).

Values presented in the different graphs are mean ± sem (standard error of the mean) or sdm (standard deviation mean). Different letters indicate statistically significant differences (LSD test; α= 0.05).

HPLC and LC-MS analysis

Quantitative analysis of stilbenes were carried out by HPLC (Pezet *et al*., 2003). A Ginkotek system (model 480) consisting of a quaternary pump, an automatic solvent degasser, an autosampler, a diode array detector, and the Chromeleon software (Dionex) was used. Qualitative analysis was performed by HPLC-MS using an Agilent 1100 series LC/MSD trap in ESI mode. Both HPLC systems were equipped with a 250 mm x 4.6 mm i.d., 5 µm, C18 Lichrospher column (Merck). The column temperature was 25 °C for HPLC-MS system and room temperature for HPLC. HPLC detection of stilbenes was at 307 nm, and calibration curves were calculated according to the standard method (injection volume 10 µL). UV spectra were made from 200 to 400 nm using a diode array online detection. For seedlings, leaf material (0.3 cm diameter) from necrotic and non-necrotic zones was collected for HPLC analysis at different time points and stored at –80°C.

Microextraction of the sample

The small leaf pieces (0.3 cm diameter) from inoculation zones and surrounding tisue were crushed with 60 µL of MeOH, transferred into a glass tube and centrifuged (ALC

Microcentrifugette 4214, Kontron, 14000 rpm). 10-30 µL aliquots of the supernatant were directly injected into the HPLC or HPLC-MS systems (Pezet *et al*., 2003).

RNA gel blot analysis

Total RNA was extracted by homogenizing frozen leaf tissue in extraction buffer (2% CTAB, 2% PVP K30, 100 mM Tris-HCl pH 8.0, 25 mM EDTA, 2 M NaCl, 0.5 g/L spermine; 5 mL/g leaf tissue). The homogenates were extracted with phenol and chloroform and the RNA was precipitated with LiCl, as described by Sambrook *et al*. (1989). For RNA blot analysis, 10 µg total RNA per sample were separated on a 1.2% agarose gel containing 5% formaldehyde, and blotted onto Hybond^{TM}-XL membranes (Amersham place, Amersham Biosciences UK limited) by capillary transfer. The RNA blot was hybridized at 65° C with cDNA probes (stilbene synthase gene (*STS-1*), Sparvoli et al., 1994) labeled with α-^{32}P-dCTP by random primer labeling. Blots were exposed for 24 h on X-Omat AR film (Kodak).

RESULTS

Development of *P. viticola* and necrosis in grapevine with and without BABA treatment

White sporulation of *P. viticola* was reduced in BABA-treated leaf discs as well as seedlings (Fig. 1), compared to water-treated control plants (Fig. 1A). Necrosis surrounding the infection sites was observed in BABA-treated seedlings (Fig. 1B) and discs (Fig. 1D) infected with *P. viticola*. Necrosis was higher in BABA-treated Solaris than in controls. Hyphal growth was observed 7 hours after inoculation. Extensive hyphal growth and spread were observed within 3 days in water-treated control plants (Fig. 1E). However, hyphal growth and spread of *P. viticola* were reduced in BABA-treated Chasselas (Fig. 1F). The number of sporangia on a

single sporangiophore was much higher in water-treated plants (Fig. 1G), whereas a very low number of sporangia on distorted sporangiophores was observed in BABA-treated plants (Fig. 1H). While sparse sporulation was observed in non-treated Solaris, no infection was observed in BABA-treated Solaris.

Phytochemical reactions at the cellular level in BABA-treated plants

The biochemical changes at the cellular level were analysed at different time points (1-5 dpi) in BABA-treated plants by using different staining techniques. Accumulation of resveratrol and flavonoids was not observed in infected water-treated plants (Fig. 1I, 1K). Blue fluorescence for resveratrol (Fig. 1J) and yellow fluorescence for flavonoids (Fig. 1L) was observed in BABA-treated plants upon infection. Resveratrol was observed at the cellular level after one day of inoculation. Accumulation of resveratrol and flavonoid compounds, callose deposition and lignification increased with time in BABA-treated plants (Chasselas and Solaris) following infection with *P. viticola* (Table 1).

Involvement of phenolic compounds in BABA-IR

The involvement of stilbenes in BABA-IR was assessed by HPLC and/or HPLC-MS methods. The leaf discs were treated with BABA, inoculated one day later and the necrosis was observed after 6 days of inoculation in treated plants. Significant protection was found in BABA-treated seedlings (Fig. 1B) as well as in leaf discs (Fig. 1D). Stilbenes were quantified from the leaf discs at the inoculation zones (Fig. 2A, 2C) as well as outside of the inoculation zones (Fig. 2B, 2D). In Chasselas, induction of stilbenes, especially of *trans*-resveratrol was higher at the inoculation zones in BABA-treated leaf discs than in control discs following infection, while accumulation level of stilbenes were similar outside of inoculation zones in

both treated and control discs. Generally, stilbene accumulation was higher at the inoculation zones than outside in Chasselas (Fig. 2A, 2B), whereas it was the reverse in Solaris (Fig. 2C, 2D). Stilbenes were below detection level in non-infected water-treated discs in both Chasselas and Solaris.

In seedlings, leaf tissues were collected randomly from water-treated control plants as well as from both necrotic and non-necrotic zones in BABA-treated plants. Induction of *trans*-resveratrol was below detection level in non-inoculated control plants, whereas *trans*-ε-viniferin was below detection level in water-treated control plants (both without and with *P. viticola*) (Fig. 3A). Induction of stilbenes was high in leaves of BABA-treated plants challenged with *P. viticola*, but very low in non-inoculated plants in both treated and control plants. Accumulation of *trans*-resveratrol was primed in BABA-treated plants at both necrotic and non-necrotic zones following infection with *P. viticola*. Interestingly, accumulation of *trans*-ε-viniferin was much higher at the necrotic zones than non-necrotic zones in BABA-treated plants inoculated with *P. viticola*.

Toxic effect of stilbenes on the mobility of zoospores

To assess their toxicity on *P. viticola*, the mobility of zoospores was tested in presence of different viniferins and *trans*-δ-resvepterol (Fig. 3B). Different concentrations (0.001, 0.01, 0.1, 1 mM) of chemicals were used. The number of mobile zoospores was significantly lower

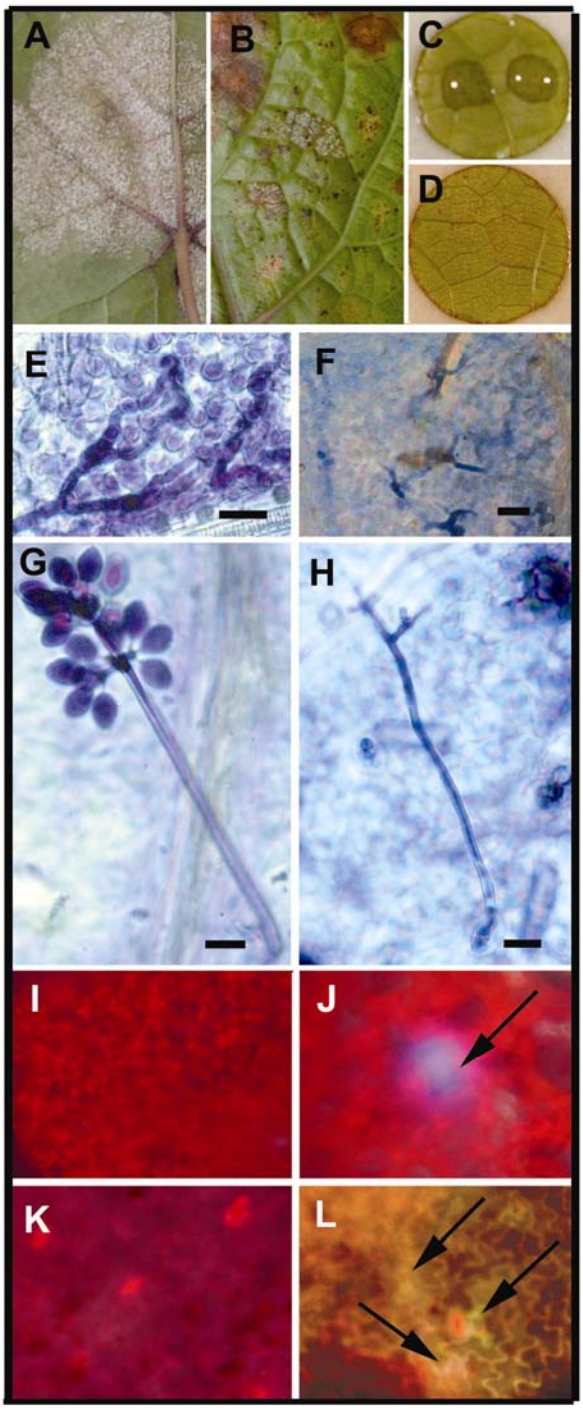

Fig. 1. Development of *Plasmopara viticola*, necrosis and accumulation of stilbene compounds in grapevine (Chasselas). **A** and **B** are leaves of seedlings; seedlings were soil-drenched with water (A) or 1 mM BABA (B) 2 days prior to challenge inoculation with sporangia of *P. viticola* ($5x10^4$ sporangia mL^{-1}). **C** through **L** , leaf discs (1 cm diameter) were punched from the third and fourth leaves from the top of a plant. Discs were treated with water (C, E, G, I, and K) or 1 mM BABA (D, F, H, J, and L) 24 hours prior to challenge inoculation with sporangia of *P. viticola* ($5x10^4$ sporangia mL^{-1}). **A**, white sporulation of *P. viticola* on water-treated seedlings (6dpi); **B**, development of necrosis surrounding infection sites in BABA-treated seedlings (6dpi). **C**, inoculation zones (two 10 µL drops of inoculum per disc) on leaf disc; **D**, BABA-treated leaf disc 6 days post inoculation (dpi). Leaf discs were stained with lactophenol-trypan blue and analysed by light microscopy (E, F, G, and H), or autofluorescence (I and J), or stained with Wilson's reagent and analysed by epifluorescence microscopy (K and L). **E**, intercellular hyphae of *P. viticola* in non-treated control (3 dpi); **F**, reduced intercellular hyphae surrounded by necrosis in BABA-treated disc (3 dpi); **G**, sporangiophore with a bunch of sporangia in non-treated control (6 dpi); **H**, distorted sporangiophore with very low number of sporangia following BABA treatment (6 dpi). **I** (non-treated control, 2 dpi), no resveratrol accumulation (red fluoresced chlorophyll in normal host tissues); **J** (BABA-treated, 2 dpi), blue fluoresced resveratrol in tissues (arrow head). **K** (non-treated control, 3dpi), no flavonoids accumulation (red fluoresced chlorophyll in normal host tissues); **L** (BABA-treated, 3 dpi), yellow fluoresced flavonoids in cell walls (arrows head). Bar = 20 µm.

(approximately 50%) at higher concentrations (0.01, 0.1, 1 mM) of *trans*-ε-viniferin, *trans*-δ-viniferin and *trans*-δ-resvepterol compared to control solutions. On average, the toxic effect of *trans*-δ-resvepterol was higher than *trans*-ε- and *trans*-δ-viniferin at lower concentrations (0.01, 0.1 mM). Less than 25% of zoospores were mobile at the highest concentration (1mM) of the chemicals. However, in highest concentration (1 mM) the number of mobile zoospores was statistically the same in different stilbenes.

Table 1. Autofluorescence, callose, lignin, flavonoids, resveratrol and gallic acid derivatives formation in grapevine infected with *Plasmopara viticola*.

Reagent	Treatment		Magnitude of reaction at different days after inoculation (DAI)					Interpreta-tion
			1	2	3	4	5	
Autofluorescence (no treatment)	Chasselas	Water	-	-	-	-	-	Blue = Resveratrol
		BABA	Blue (B)	B+	B++	B++	B+++	
	Solaris	Water	Blue (B)	B+	B++	B++	B++	
		BABA	Blue (B)	B+	B++	B+++	B+++	
Aniline blue	Chasselas	Water	-	-	-	-	-	Light green = Callose
		BABA	Green (G)	G+	G++	G+++	G+++	
	Solaris	Water	Green (G)	G+	G++	G++	G+++	
		BABA	Green (G)	G+	G++	G+++	G+++	
Phloroglu-cinol-HCL	Chasselas	Water	-	-	-	-	-	Red = Lignin
		BABA	Red (R)	R+	R++	R++	R+++	
	Solaris	Water	Red (R)	R+	R++	R++	R+++	
		BABA	Red (R)	R+	R++	R+++	R+++	
Wilson's Reagent	Chasselas	Water	-	-	-	-	-	Yellow = Flavonoids; Blue = Gallic acid derivative (GAD)
		BABA	Yellow (Y)	Y+	Y++	Y++	Y+++	
			Blue (B)	B+	B++	B++	B+++	
	Solaris	Water	Yellow (Y)	Y+	Y++	Y++	Y+++	
			Blue (B)	B+	B++	B++	B+++	
		BABA	Yellow (Y)	Y+	Y++	Y+++	Y+++	
			Blue (B)	B+	B++	B+++	B+++	

BABA = β-aminobutyric acid (1mM); Y = Yellow, B = Blue, G = Green, R = Red ;

+ = > +++, increasing magnitude of response; -, no response

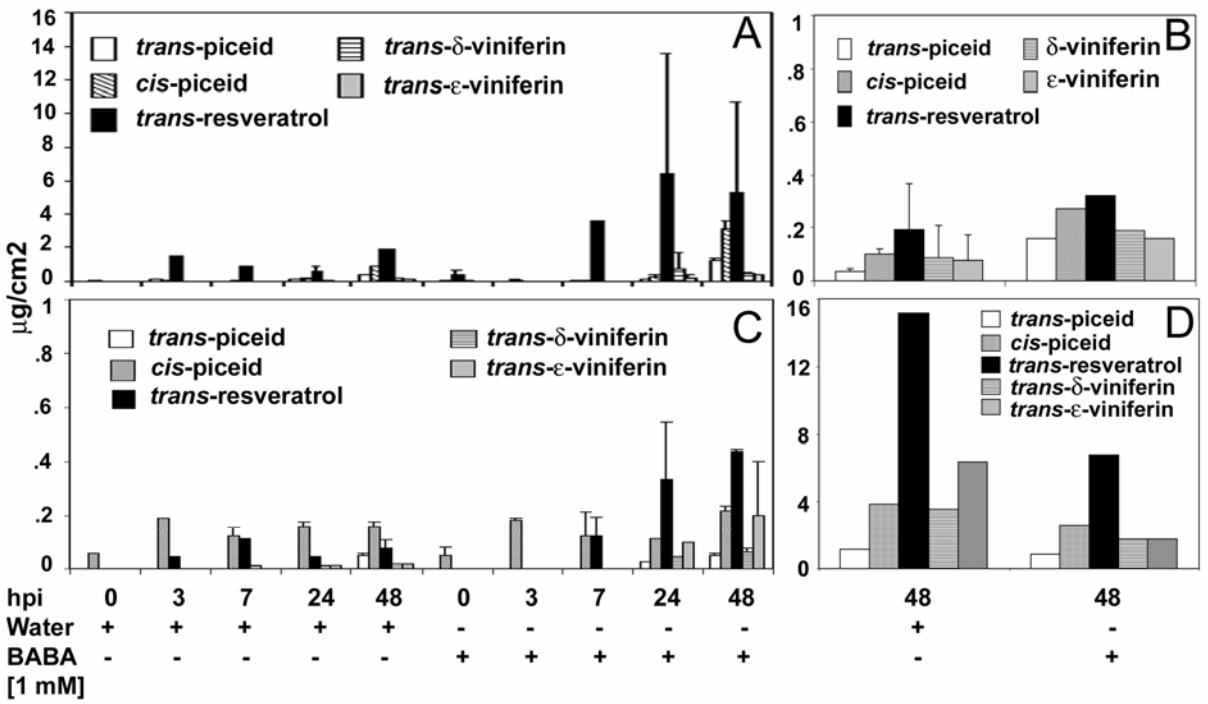

Fig. 2. Accumulation of stilbenes (µg/cm^2 leaf area) at the inoculation zones and outside of the inoculation zones in grapevine following infection with *Plasmopara viticola*. **A** through **D**, leaf discs (1 cm diameter) of grapevine (A and B are susceptible variety Chasselas, and C and D are resistant variety Solaris). Quantification of different stilbenes, **A** and **C**, at the inoculation zones; **B** and **D**, at the outside of the inoculation zones. Leaf discs (12 discs/treatment ; 3 replications/treatment) were punched from the third and fourth leaves from the top of a plant. Leaf discs were treated with water or BABA (1 mM) 24 hours prior to challenge inoculation with sporangia of *P. viticola* (5x10^4 sporangia mL^{-1}). Small pieces (0.3 cm diameter) of cut out leaf material (2 pieces/disc, 4 leaf discs/treatment) were collected from the different inoculation zones also from the non-inoculation zones as described in materials and methods. Stilbenes were analyzed by using high-performance liquid chromatography (HPLC). Values presented are means ± sdm (standard deviation mean). The experiments were repeated 2 times with similar results. hpi: hours post inoculation.

Role of stilbene synthase gene in BABA-IR

To elucidate the involvement of a stilbene synthase gene the expression profile of *STS-1* was analysed. Expression of *STS-1* was higher in JA-treated seedlings (Chasselas) than in BTH-treated plants (Fig. 4A). *STS-1* expression was low in ABA-treated and water-treated control plants. The Northern blot result showed that *STS-1* was expressed in BABA-treated plants following infection with *P. viticola* (Fig. 4B). *STS-1* expression started 7 hpi with a peak at 24 hpi in BABA-treated plants whereas no expression was observed in infected water-treated control plants.

DISCUSSION

Enhancement of plant defense mechanisms is assumed to be a good tool for combatting plant disease and an alternative environment-friendly approach. In this study, BABA is shown to be an efficient inducer of multiple defense responses in grapevine, including accumulation of stilbenes and potentiation of *STS-1* expression upon infection with *P. viticola*.

Leaf discs as well as seedlings treated with BABA showed necrotic reactions following infection with *P. viticola*. Necrosis was observed surrounding the infection zones. The involvement of phenolics and stilbenes was investigated at the inoculation zones and in the surrounding tissue. In staining experiments, accumulation of resveratrol was observed in BABA-treated plants upon infection. Accumulation of resveratrol was quantified by HPLC. *trans*-resveratrol induction was very high in BABA-treated plants upon infection and very low in water-treated controls.

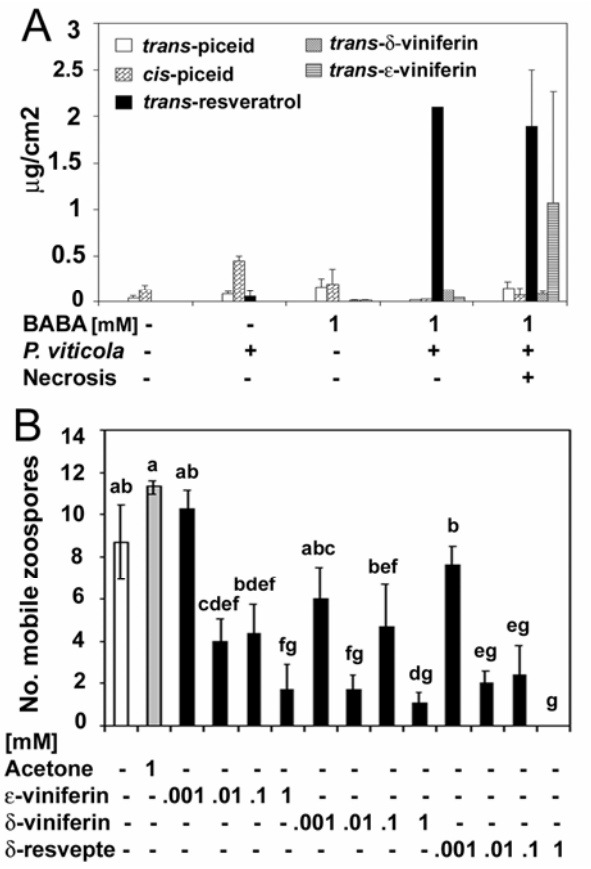

Fig. 3. A, accumulation of stilbenes (µg/cm^2 leaf area) in seedlings of grapevine (Chasselas) upon infection with *Plasmopara viticola*. Seedlings were taken at the 4-5 leaf stage. The plants were soil-drenched with water or BABA (1 mM) 2 days prior to challenge inoculation with sporangia of *P. viticola* (5x10^4 sporangia mL^{-1}). Small pieces (0.3 cm diameter) of cut out leaf material (2 pieces/leaf, 4 leaves/treatment) were collected from the different necrotic zones and also from the non-necrotic zones as described in materials and methods. Stilbenes were analyzed by HPLC. **B**, toxic effect of stilbenes (*trans*-ε-viniferin, *trans*-δ-viniferin and *trans*-δ-resvepterol) on the mobility of zoospores of *P. viticola* (10^5 sporangia mL^{-1}). Different stilbene solutions (dissolved in 1% acetone) were added into zoospores containing suspensions and the number of live zoospores was quantified in an area of 0.11 mm^2 under the microscope as described in materials and methods. Values presented are means ± sem (LSD test ; α= 0.05). The experiments were repeated 2 times with similar results. δ-resvepte: *trans*-δ-resvepterol.

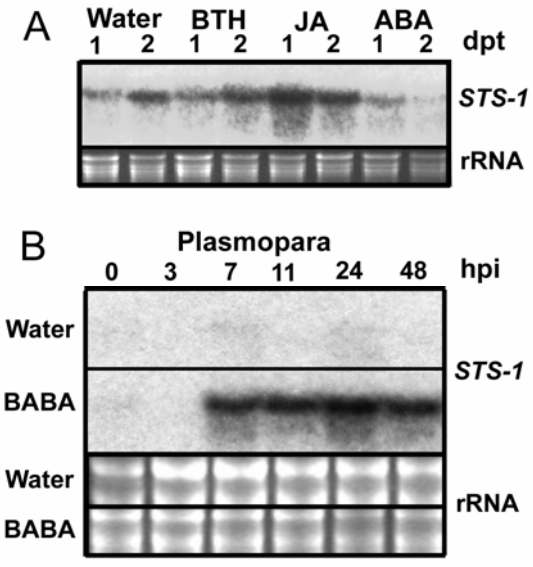

Fig. 4. Expression pattern of the stilbene synthase gene (*STS-1*) in seedlings of grapevine (Chasselas) upon treatment with different inducers (A) and infection with *Plasmopara viticola* (B). **A**, the seedlings were soil-drenched with water, BTH (0.5 mM), JA (0.1 mM) or ABA (0.1 mM); **B**, the seedlings were soil-drenched with water or BABA (0.5 mM) 2 days prior to challenge inoculation with sporangia of *P. viticola* (5x10^4 sporangia mL^{-1}). Total RNA was extracted at different time points after treatment (1 and 2 dpt) as well as after infection. Each time-point represents 1 g of leaf materials (8 leaves from 4 different plants). RNA blots were hybridised with *STS-1* probes. Ethidium bromide staining of the RNA gel (rRNA) was used to show equal loading. The experiments were repeated twice with similar results. dpt: days post treatment. hpi: hours post inoculation.

Langcake and Pryce (1976) identified *trans*-resveratrol as a major component responsible for the blue fluorescence of grapevine leaf tissues upon infection with *P. viticola*. Recent studies suggested to consider *trans*-resveratrol as a phytoalexin because of its toxicity against *Botrytis cinerea* (Adrian et al., 1997, 1998), *P. viticola* (Dercks and Creasy, 1989; Dai et al., 1995) or *Phomopsis viticola* (Hoos and Blaich, 1990). The presence of *trans*-resveratrol in the necrotic tissues in resistant varieties of grapevine might restrict the hyphal development of *P. viticola* (Dai et al., 1995). Recently, Montero and colleagues (2003) reported that in *B. cinerea*-infected grapes, ethylene emission rises after 48h when the *trans*-resveratrol starts to decrease irreversibly, whereas in non-infected grapes a high *trans*-resveratrol content corresponds to a low ethylene emission. They also suggested that exogenous application of *trans*-reveratrol has a positive effect on fruit conservation during storage and that it doubled the normal shelf-life of grapes. Necrosis was observed in BABA-treated plants upon infection with *P. viticola*. In Chasselas, the higher accumulation of phenolics and stilbenes at the inoculation zones compared to the area surrounding revealed the local reaction to *P. viticola* infection in BABA-treated leaf discs. This localized reaction might contribute to the cell death around infection sites. In contrast, outside of inoculation zones the accumulation of *trans*-resveratrol was higher in water-treated than in BABA-treated Solaris. Less infection may cause lower accumulation of stilbenes in BABA-treated Solaris.

In seedlings, *trans*-resveratrol accumulation was higher in non-necrotic than in necrotic zones in BABA-treated plants upon infection. In contrast, *trans*-ε-viniferin accumulation was higher in the necrotic area compared to the non-necrotic zones. This suggests that a part of the accumulated resveratrol in the non-necrotic zones was oxidized and converted into *trans*-ε-viniferin which might be one of the causes of necrosis. This supports observations that extracellular ε-viniferin could result from an oxidative dimerization of resveratrol triggered by

a cell wall-localized peroxidase (Calderon et al, 1992, 1994; Langcake and Pryce 1977a). δ-viniferin is an isomer of ε-viniferin produced *in vitro* by the oxidative dimerization of resveratrol by plant peroxidases or fungal laccases (Pezet et al., 2003).

Pezet and colleagues (2004) reported that the different stilbenes are toxic for zoospores of *P. viticola*. This could affect the disease development in grapevine. High concentration of stilbenes at the site of infection in the resistant variety Solaris correlated with inhibition of *P. viticola* development. In contrast, the observed resistance in different grapevine varieties to *P. viticola* may be associated with some factors other than stilbenes (Dercks and Creasy, 1989). The toxicity of stilbenes was concentration-dependent, but all stilbenes like *trans*-δ-viniferin, *trans*-ε-viniferin and *trans*-δ-resvepterol inhibited the mobility of zoospores at high concentration. Kortekamp et al. (1998) reported that fungal development was stopped three days after inoculation in resistant cultivars, whereas infection of leaves was similar to that observed in susceptible cultivars. This indicates that after the start of infection phytoalexins might accumulate in resistant cultivars, stopping further development and late infection. We show that germination as well as penetration of the zoospores is not simultaneous. Therefore, zoospores that try to penetrate might well be stopped by the accumulated phytoalexins induced by an earlier penetration event. This might prevent further infection.

In the staining experiment, we observed that stilbenes accumulated in the adjacent cells surrounding the penetration site. Stilbenes were extracted from small pieces (0.3 cm diameter) of leaf discs excised from the inoculation zones as well as from the necrotic zones. Stilbene contents were expressed as $\mu g/cm^2$ leaf area. We found that stilbenes were not evenly accumulating all over the tissues, but rather in a limited number of cells. This means that the actual concentration of the different stilbenes was much higher in the responding cells than what we measured and this higher level of accumulation even more toxic to the zoospores.

Aziz and colleagues (2003) reported that large amounts of resveratrol and viniferin were produced by grapevine cells in response to laminarin treatment. The expression of a stilbene synthase gene (*STS-1*) had a peak at 5 hpi in laminarin-treated cells and slowly declined. Repka (2002) showed that bestatin primes resveratrol accumulation and the expression of PAL gene in grapevine cells.

Synthesis of stilbenes only occurs if PAL and stilbene synthase genes are induced (Jeandet et al., 2002). Coutos-Thevenot and colleagues (2001) showed that genetic transformation of grapevine with the chimeric PR-10 promoter-Vst1 (vitis stilbene synthase 1) construct increased resveratrol production during fungal infection. Expression of *STS-1* was high in JA-treated plants indicating the involvement of the JA signal transduction pathways. *STS-1* induction started 7 hpi at the time of penetration of zoospores into stomata in BABA-treated plants following infection with *P. viticola*. The potentiated expression of *STS-1* peaked at 24 hpi. This result is supported by our previous report (Hamiduzzaman et al., 2005) showing that BABA-IR acts through the JA signaling pathways in grapevine. Activation of the *STS-1* gene in BABA-treated plants led to the accumulation of phenolics and stilbenes like *trans*-resveratrol within plant tissues upon infection. The results presented here suggest that BABA-IR against *P. viticola* in grapevine involves priming of the JA signal transduction pathways leading to potentiated accumulation of stilbenes.

Acknowledgements

We thank RAC Changin for grapevine plants. We also thank Bettina Gsell for seeds of grapevine. This project was funded by the National Centre of Competence in Research (NCCR) Plant Survival, research programme of the Swiss National Science Foundation.

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CHAPTER 5

General discussion

Plants have the ability to develop an enhanced defensive capacity upon challenge with pathogenic or non-pathogenic microorganisms. One possibility of defense is induced resistance, generally expressed as a restriction of growth of the pathogen and a reduction of disease symptoms (Hammerschmidt, 1999). Plant defense responses are controlled by multiple signal transduction pathways where SA, JA and ethylene are involved. These regulators control and potentiate the activity of distinct defense pathways helping the plant to prioritize a specific activity in response to single or multiple stresses (Reymond and Farmer, 1998; Glazebrook, 1999; Pieterse and Van Loon, 1999). The different signaling pathways controlling SAR and ISR are well studied in *Arabidopsis*. Pathogen-induced SAR depends on endogenous accumulation of SA and is associated with the induction of pathogenesis-related proteins (Ryals et al., 1996; Sticher et al., 1997; Van Loon, 1997). It has been reported in some cases that rhizobacteria could also activate the SAR pathway by producing SA at the root surface (Maurhofer et al., 1994, 1998; De Meyer and Höfte, 1997; De Meyer et al., 1999).

In grapevine, the expression of genes of the phenylpropanoid pathway was induced by SAR activators suggesting a role for CCoAOMT and stilbene synthase in the disease-resistance reaction (Busam et al., 1997a). ß-aminobutyric acid, a non-protein amino acid has a broad spectrum of protective activity in many plant species against oomycetes (Cohen, 2000; Cohen et al., 1994) and necrotrophic fungi (Ton and Mauch-Mani, 2004) in Arabidopsis, downy mildew in grapevine (Cohen et al., 1999), lettuce (Pajot et al., 2001) and cauliflower (Silue et al., 2002). BABA not only protects plants from biotic stresses, it is also effective against abiotic stresses such as salt, cold or drought stress in Arabidopsis (Conrath et al., 2002; Jakab et al., 2005).

Most of the studies about defense responses observed in grapevine deal with gene expression, activities of chitinases and glucanases (Busam et al., 1997; Robinson et al., 1997; Robert et al., 2002), or production of phytoalexins (Pryce and Langcake, 1977; Adrian et al., 1997; Bais et al., 2000). Recent reports show that an endopolygalacturonase of *B. cinerea* (Poinssot et al., 2003) and laminarin (Aziz et al., 2003) induce other defense responses in grapevine. Unlike other plants (eg. Arabidopsis), there were no marker genes available for SA, JA and ABA signal transduction pathways in grapevine. To be able to further study the involvement of these defense pathways during BABA-induced resistance (BABA-IR) in grapevine, the research described in this thesis was mainly focussed on elucidating the following questions:

-What are the components playing a role in the establishment of BABA-IR in grapevine against *P. viticola*?

-Is there any involvement of phytoalexins in BABA-IR?

-What are the defense signaling pathways primed by BABA?

Components involved in BABA-IR

Several approaches were initiated to identify the components involved in BABA-IR in grapevine. Leaf disc assay results showed great differences in the reactions of the two varieties of grapevine following infection with downy mildew. Sporulation of *P. viticola* was reduced in BABA- and JA-treated compared to BTH- and ABA-treated grapevine. Necrosis was observed around the infection sites in both BABA-treated Chasselas and Solaris. Gindro et al. (2003) suggested that germ tubes of zoospores of *P. viticola* cannot penetrate the stomata because of rapid defense responses by callose accumulation in the resistant cultivar Solaris. We observed that biochemical changes at the cellular level in BABA-treated plants occurred only upon infection with *P. viticola*, not in non-inoculated BABA-treated plants, revealing that BABA acts via priming. As stomata are very important for *P. viticola* infection, callose induction in the substomatal cavity, lignification around the infection sites and in guard cells of stomata, and flavonoid at the cell walls might restrict its growth and development in BABA-treated plants. This results support earlier reports where callose deposition was observed in BABA-treated Arabidopsis upon challenge with *Hyaloperonospora parasitica* (Zimmerli et al. 2000). Callose deposition was enhanced in BABA-treated cauliflower leaves around haustoria developed by *H. parasitica* and lignin was accumulated in BABA-treated grapevine following inoculation with *P. viticola* (Cohen 2002). To elucidate the role of callose and lignin in BABA-IR, different inhibitors (AIP, a PAL inhibitor; DDG, a callose inhibitor) were used. In Chasselas, callose accumulation decreased after infection when DDG was applied together with BABA. Lignification also decreased with the co-application of AIP. In non-treated Solaris, callose and lignin accumulated upon infection with *P. viticola*, which may contribute to the basal resistance of the plants. Mauch-Mani and Slusarenko (1996) reported that PAL is involved in synthesizing SA and precursors for lignification in Arabidopsis, and

the PAL-inhibitor AIP made plants completely susceptible to *H. parasitica*. Dai et al. (1995) observed necrosis of cell walls and guard cell of stomata in resistant grapevine. They suggested that accumulation of flavonoids and lignin might reduce the infection of *P. viticola* in a resistant variety. DDG together with AIP reduced BABA-IR in Chasselas and basal resistance in Solaris significantly, indicating the synergistic effect of callose and lignin in defense reactions. However, this did not result in a susceptibility comparable to water-treated Chasselas plants. This results support earlier reports that DDG reduced BABA-IR as well as callose deposition in Arabidopsis against *A. brassicicola* (Ton and Mauch-Mani 2004). They showed that reduction of callose accumulation correlated with increased lesion size.

Role of stilbenes in BABA-IR

Necrosis was found around infection sites in BABA-treated plants following infection with *P. viticola*. The presence of higher amount of *trans*-resveratrol and *trans*-ε-viniferin at inoculation zones as well as at necrotic areas in BABA-treated plants suggested the involvement of stilbenes in local necrotic-like reactions. Phytoalexins have been shown to be important natural compounds in the defense of plants against fungal infection (Jeandet et al. 2002, Kuc 1995). Several crops, including grapevine and peanut, synthesize the stilbene-type phytoalexin resveratrol (trans-3,4,5-trihydroxy-stilbene) when attacked by pathogens (Langcake and Pryce 1977). Stilbene synthesis is induced in grapevine by inoculation with the pathogens *B. cinerea* or *P. viticola* (Blaich and Bachmann 1980, Langcake 1981). BABA primed stilbene accumulation in grapevine was correlated with the reduced sporulation of *P. viticola* in BABA-treated plants. This supports earlier report that the subsequent level of resistance to *P. viticola* was positively correlated with the capacity of grapevine to synthesize stilbene (Dercks and Creasy 1989). Resveratrol is synthesized by the enzyme

trihydroxystilbene synthase (stilbene synthase or resveratrol synthase), using as substrates one molecule of 4-coumaroyl-CoA plus three molecules of malonyl-CoA (Rupprich and Kindl 1978). Since these two substrates are commonly present in plants, introduction of a single gene encoding stilbene synthase may be sufficient to synthesize resveratrol in heterologous plant species for increased resistance against fungal pathogens (Zhu et al. 2004).

The toxic effect of different stilbenes on the mobility of zoospores was studied. Pezet et al. (2004) reported that the different stilbenes have a toxic effect on the mobility of zoospores of *P. viticola*, which affects the disease development in grapevine. We assume that stilbenes accumulate in tissues adjacent to the sites of penetration, diffuse to the surface and remain there. *In vivo* presence of stilbenes might reduce the number of live zoospores on the leaf surface. Dai et al. (1995) proposed trans-resveratrol to be related to diffuse necrosis and to inhibition of *P. viticola* in resistant grapevine. We show that induction of stilbenes was higher in BABA-treated plants than in non-treated controls, which enhanced the defense activity of the plants. Furthermore, we observed that stilbenes accumulated in the adjacent cells limited at the infection sites rather than all other leaf tissues, suggesting that actual concentrations of stilbenes at the sites of penetration were much higher than measured from leaf patches. Further studies are needed to elucidate the biochemical and molecular mechanisms of both the localization and metabolism of stilbenes during BABA-IR in grapevine.

Potentiation of signaling pathways in grapevine

To elucidate the mechanisms behind BABA-IR against *P. viticola*, we examined the involvement of SA-, JA- and ABA-dependent defenses. The highest protection against *P. viticola* was observed in BABA- and JA-treated plants while it was low after BTH and ABA-treatment. Callose accumulation was also higher in BABA- and JA-treated than in BTH- and

ABA-treated plants. It has been reported that the presence of high concentrations of ABA made the plant even more susceptible to Pierce's disease in grapevine (Lu et al., 2001) and to *B. cinerea* in tomato (Audenaert et al., 2002). In contrast, ABA regulated plant responses to abiotic stresses (Xiong et al., 2002) as well as induced resistance against *Plectosphaerella cucumerina* and *Alternaria brassicicola* (Ton and Mauch-Mani, 2004). Anderson et al. (2004) suggested a novel role for ABA in modulating defense gene expression and disease resistance in Arabidopsis. They reported that endogenous and exogenous ABA strongly reduced the transcript levels of JA-ethylene responsive defense genes. Exogenous methyl jasmonate or ethylene was not able to reverse this suppression caused by the ABA treatment in wild-type plants. The antagonistic interaction between the ABA and the JA-ethylene signaling pathways in regulating defense gene expression might be a strategy that plants employ to avoid simultaneous production of abiotic stress-related and biotic defensive proteins.

To investigate the involvement of different signal transduction pathways in BABA-IR in grapevine, the expression pattern of defense-related marker genes was investigated. *PR-1* was highly expressed in both water and BABA-treated leaf discs, whereas *PR-4* and *LOX-9* showed potentiated expression in BABA-treated leaf discs following *P. viticola* infection. Experiments with Arabidopsis mutants affected in JA biosynthesis or signaling showed they are more susceptible to *Pythium mastophorum* (Vijayan et al., 1998) as well as to insect herbivory (McConn et al., 1997; Heil and Bostock, 2002). This points to an involvement of the JA-signaling pathway in defense . Exogenous jasmonate induces genes associated with phytoalexin biosynthesis (Choi et al. 1994), antifungal thionins (Andresen et al. 1992) and osmotin (Xu et al. 1994), as well as different enzyme involved in plant defense reactions such as chalcone synthase (CHS) and phenylalanine ammonia-lyase (PAL, Creelman and Mullet 1997, Gundlach et al. 1992). Repka et al. (2001) reported that application of methyl jasmonate

to the surface of grapevine leaves caused the formation of lesions that mimic a typical hypersensitive response, stimulated SA production and expression of defense-related genes. Weak expression of the callose synthase gene (*CS*) was observed at 7 hpi in BABA-treated plants upon infection with *P. viticola* (data not shown). The similarity between BABA- and JA-induced resistance against *P. viticola* based on high callose deposition indicates that callose deposition might depend on the JA signaling pathway. In contrast, Ton et al. (2005) observed BABA-primed callose deposition in Arabidopsis depends on the ABA signaling pathway. However, further studies are needed to clarify the relationship between the expression of the callose synthase gene and JA signaling pathway.

Aziz et al. (2003) observed that *LOX*, *GST*, *PAL*, *STS1*, *CHIT4c* and *CHIT1b* genes were activated rapidly in grapevine after laminarin treatment, whereas *CHIT3*, *PIN* and *GLU1* genes were up-regulated later. Accumulation of PR proteins was potentiated in BABA-treated Arabidopsis upon inoculation with *Pseudomonas syringae* (Zimmerli et al. 2000), *Botrytis cinerea* (Zimmerli et al. 2001), and in cauliflower against *P. parasitica* (Silue et al. 2002). In contrast, accumulation of PR proteins was found in non-infected BABA-treated tobacco and tomato plants (Jeun 2000, Cohen 2002). The expression of *PR-1* was observed in both the water- and BABA-treated grapevine without inoculation. The low protection of *P. viticola* with BTH and ABA and the unclear expression pattern of *PR-1*, rule out any major involvement of SA and ABA signal transduction pathways. Furthermore, potentiated expression of *PR-4* and *LOX-9* in treated plants indicates the involvement of the JA signaling pathway.

Powdery mildew (*Uncinula necator*) is also an important disease of grapevine. Gianakis et al. (1998) reported a correlation between the activities of chitinase and glucanase of different grapevine cultivars and their resistance to powdery mildew. The effect of powdery mildew

infection on the expression of defense-related genes was studied in comparison to downy mildew infection. *PR-1* and *PR-4* genes were highly induced in powdery mildew infected leaves, while expression was low and transient in downy mildew infected leaves. *LOX-9* expression was observed in neither powdery nor in downy mildew infected leaves, whereas the expression of *OSM-1* was observed in both cases. This supports earlier reports that JA plays an important role in the transcription of osmotin genes following wounding in Arabidopsis (Titarenko et al., 1997) and in potato (Zhu et al., 1995). We studied the expression of defense-related genes in grapevine at different time points after infection with *P. viticola*, in the case of powdery mildew, the expression was analyzed on the basis of different symptoms and necrosis from naturally infected leaves of grapevine. Further studies will be needed to clarify the expression pattern of defense-related genes and the involvement of signaling pathways at different time points after powdery mildew infection.

The influence of leaf age on defense reactions in grapevine was analyzed. In older leaves, induction of marker genes for the SA signaling pathway rather than the JA signaling pathway suggests the involvement of the former pathway in age related resistance. Kus and colleagues (2002) already reported that ARR is dependent on SA signaling in Arabidopsis and SA acted as a signal for the production of antimicrobial compound against *P. syringae*. Ward et al. (1981) observed a positive correlation between increasing plant age, glyceollin production and resistance to *Phytophthora megasperma* in soybean.

Wounding, an abiotic stress, induces different types of defense-related genes in tomato (Chen et al., 2004), wheat (Igawa et al., 2004) and hybrid poplar (Christopher et al., 2004). The transcription of osmotin-like genes was induced by wounding in potato plants (Zhu et al., 1995), while the expression of the PIN genes was shown to be induced by JA in tomato (Doares et al., 1995) and by wounding in maize plants (Tomayo et al., 2000). In grapevine,

compared to other plants, wounding did not elicit active oxygen species (AOS) production (Papadakis and Roubelakis-Angelakis, 1999), but induced the stress metabolite resveratrol (Becker et al., 2003). The effect of aging and wounding was studied on defense responses in seedlings. *PR-1* expression was very high in wounded water-treated seedlings while potentiated expression of *PR-4* and *OSM-1* was observed in wounded BABA-treated seedlings. No effect of wounding was found on *LOX-9* expression. In Arabidopsis, BABA-primed resistance against abiotic (salt) stress was based on the ABA-regulated defense pathway (Ton et al. 2005). Our results suggest that older, necrotic leaves as well as wounding of leaves, induces defense-related genes in grapevine.

Involvement of *STS-1* and JA signaling pathways in BABA-IR

Aziz and colleagues (2003) reported that large amounts of resveratrol and viniferin were produced, and the expression of a stilbene synthase gene (*STS-1*) peaked at 5hpi in laminarin-treated grapevine cells and then slowly declined. Products of *PAL* and *STS1* are involved in the regulation of metabolic flux into the pathway for the synthesis of stilbenic phytoalexins, such as resveratrol, which exert antifungal activity against *B. cinerea* (Adrian et al. 1997, Bais et al. 2000). Induction of *PAL* and *STS* genes correlated with enhanced synthesis of stilbene in plants (Jeandet et al. 2002).

Stilbene synthase genes isolated from grapevine have been transformed into different plants. Grapevine, tobacco and tomato transformed with *Vst1* (Vitis stilbene synthase 1) under the control of its own promoter showed a rapid accumulation of both stilbene synthase mRNA and its product stilbene, following inoculation with pathogenic fungi. A significant increase in resistance has been reported for stilbene synthase transformed tobacco plants against *B. cinerea* (Hain et al. 1993), tomato plants against *Phytophthora infestans* (Thomzik et al.

1997), barley and wheat plants against *B. cinerea* (Leckband and Lorz 1998), and grapevine against *B. cinerea* (Coutos-Thevenot et al. 2001). Zhu et al. (2004) reported that transformation with the *Vst1* gene might increase production of resveratrol in papaya sufficiently to inhibit the *in vivo* development of *P. palmivora* to achieve increased disease resistance. However, the corresponding signal transduction pathway for expression of the *STS-1* in grapevine has not been well demonstrated.

We observed that *STS-1* expression was high in JA-treated plants indicating the involvement of the JA signal transduction pathway in stilbene accumulation. Induction of *STS-1* started 7 hpi, at the time of penetration of zoospores into stomata in BABA-treated plants. To gain further insight of the involvement of the JA pathway, the LOX inhibitor ETYA was used. Lipoxygenase inhibitors have been used to prevent wound-induced gene expression in tomato (Peña-Cortes et al., 1993), methyl jasmonate-induced gene expression in soybean (Staswick et al., 1991), and to inhibit wound- and elicitor-induced responses in parsely and tobacco (Ellard-Ivey and Douglas 1996). Veronesi et al. (1996) observed in the tobacco-*Phytophthora parasitica* var. *nicotianae* pathosystem that LOX genes were not expressed constitutively in control plants, whereas mRNA accumulated rapidly and transiently in both elicitor-treated and pathogen-inoculated plants. Repka (2002) reported that bestatin- (an inhibitor of some aminopeptidases in plants and animals) treated grapevine augmented the sensitivity for methyl jasmonate-induced hypersensitive-like response. For this, enhanced resveratrol accumulation was associated with potentiated activation of genes encoding phenylalanine ammonia-lyase (*PAL*). BABA-IR in Chasselas as well as basal resistance in Solaris against *P. viticola* was reduced by the application of ETYA. Northern blots showed that the expression of marker genes (*PR-4*, *LOX-9*) of the JA pathway decreased following co-application of ETYA with BABA. This confirms that BABA-IR is dependent on the JA signal transduction pathway and

leads to a potentiated accumulation of callose and stilbenes in grapevine (Fig. 1). Rickauer et al. (1997) studied the role of ETYA on the expression of lipoxygenase genes in tobacco plants after infection with *P. parasitica*. They showed that different defense-related transcripts were induced in methyl jasmonate-treated tobacco plants, which were partially regulated by the LOX-catalyzed jasmonate pathway.

A proposed model is schematically represented in Figure 1 about the mechanisms of BABA-IR in grapevine against *P. viticola*. The plants were treated with BABA and challenged with *P. viticola* or by wounding. We observed lignification, the product of phenylpropanoid pathway in BABA-treated plants after infection and the expression of *PR-1* gene following wounding. Co-application of AIP was able to suppress lignification as well as BABA-IR. In the JA signaling pathway *PR-4* and *LOX-9* gene expression was potentiated in BABA-treated plants. Priming of callose deposition in BABA- and JA-treated plants was suppressed by the co-application of DDG. Expression of the JA-regulated marker genes (*PR-4*, *LOX-9*) was decreased with co-application of ETYA. Indeed, co-application of ETYA was able to suppress BABA-IR. Expression of *STS-1* gene in JA-treated plants and priming of *STS1* in BABA-treated plants were observed. Accumulation of stilbenes was induced in BABA-treated plants following infection with *P. viticola*. Our result suggest that primed callose, lignin and stilbenes together made the plant resistant against downy mildew and this type of resistance is known as BABA-IR in grapevine (Fig. 1).

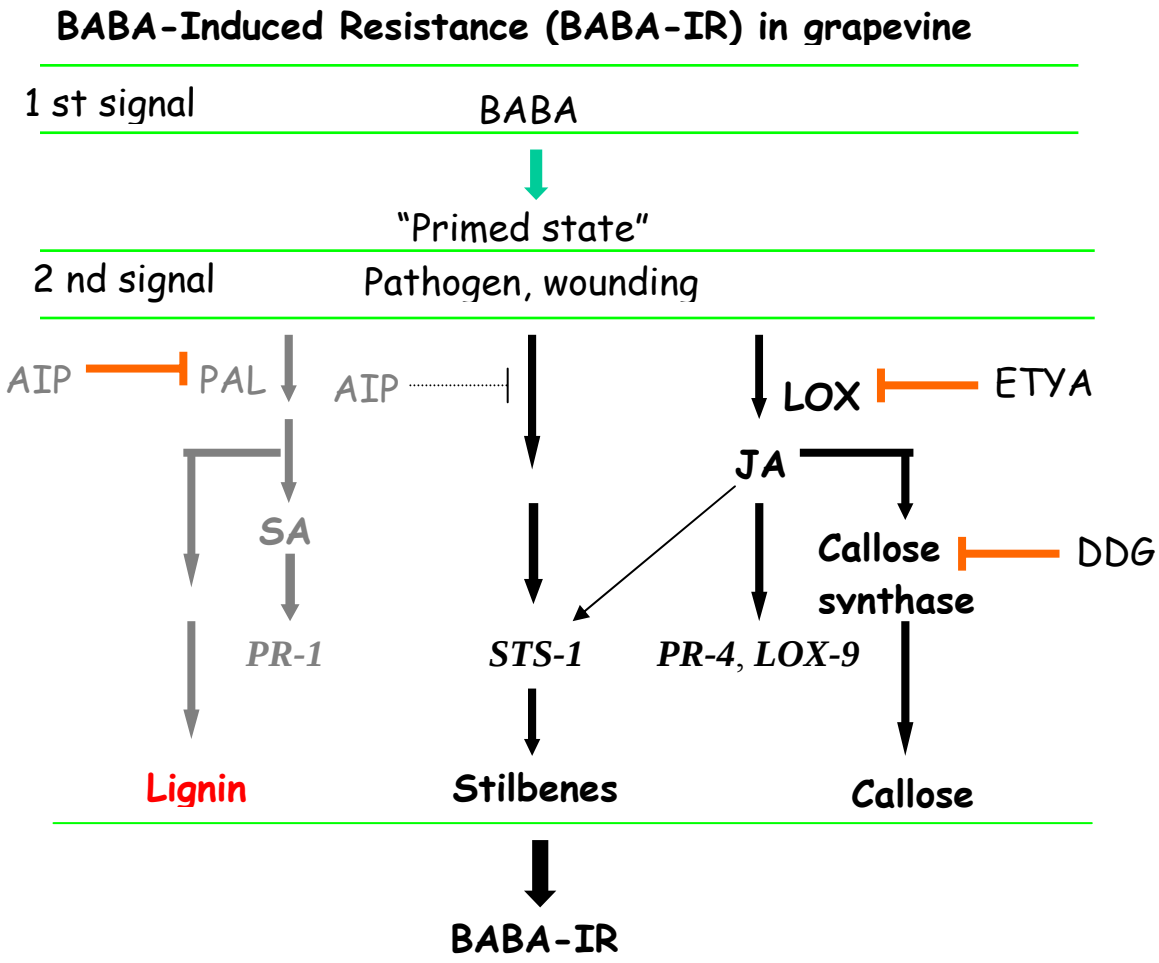

Fig. 1. Model for the mechanisms of BABA-IR in grapevine against downy mildew (*P. viticola*). Treratment with BABA induces a primed state in the plant. Upon exposure to stress (*P. viticola)* or wounding, different signal transduction pathways are activated leading finally to the accumulation of defensive or toxic product.

In conclusion, the present study shows that BABA induces resistance against *P. viticola* in a susceptible variety of grapevine. Callose deposition plays a major role in BABA- and JA-induced resistance in grapevine against downy mildew. The *LOX-9* and *PR-4* gene expression was potentiated after BABA-treatment. Future studies will be needed to clarify the relationship between the potentiation of callose synthase gene expression and the JA signaling pathway during BABA-IR in both susceptible and resistant grapevine.

Induction of *STS-1* and accumulation of stilbenes was high in BABA-treated plants after infection with *P. viticola*. Further studies will clarify how induced stilbenes prevent the penetration of *P. viticola*. In addition, the effect of the phenylpropanoid pathway on the induction of stilbenes needs further studies. Our results suggest that BABA-IR involves priming of the JA signal transduction pathway leading to callose deposition, lignification and potentiated accumulation of resveratrol and viniferins in grapevine following infection by downy mildew. To further clarify the mechanisms of induced resistance against *P. viticola* the cross-talk between SA and JA signaling pathways during BABA-IR in grapevine should be investigated.

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CURRICULUM VITAE

Dr. Mollah Md. Hamiduzzaman
Department of Plant Pathology
Bangladesh Agricultural University, Mymensingh 2202
Bangladesh.
E-mail: mmdhamid@yahoo.com
Fax: + 880-91-55810 ; Telephone: + 880 175 136 317
Date of birth: 25 June, 1968; Nationality: Bangladeshi; Marital status: Married

Educational qualification

2001- 2005 Ph.D. in Science,
Lab. of Biochemistry, Institute of Botany, University of Neuchâtel, Switzerland.

2000-2001 Research student, Plant Biology, University of Fribourg, Switzerland

1996 Master of Science (M.S.) in Plant Pathology,
Bangladesh Agricultural University, Bangladesh

1994 Bachelor of Science in Agriculture (Honours),
Bangladesh Agricultural University, Bangladesh

1985 Higher Secondary Certificate (HSC), Rajshahi Board, Bangladesh

1983 Secondary School Certificate (SSC), Rajshahi Board, Bangladesh

Experiences

Research: (i) Ph. D. research (2001 - 2005): Induced resistance in grapevine against downy mildew (*Plasmopara viticola*); Lab. of Biochemistry, Institute of Botany, University of Neuchâtel, Switzerland.
(ii) Research on induced resistance in Arabidopsis by using PGPR against *Peronospora*; Institute of Plant Biology, University of Fribourg, Switzerland (2000-2001).
(iii) Scientific Officer, Seed Pathology Centre, BAU, Mymensingh, Bangladesh (From 19 November, 1995 to 27 May, 1996).
(iv) Principal Investigator of the Project "Biological control of bacterial wilt of tomato", BAU, Bangladesh (From October, 1998 to June, 2000).

Teaching: (i) Assistant Professor, Department of Plant Pathology, Bangladesh Agricultural University, Mymensingh, Bangladesh (16 October 1998 to date).
(ii) Lecturer, Department of Plant Pathology, Bangladesh Agricultural University (BAU), Mymensingh, Bangladesh (From 28 May, 1996 to 15 October, 1998).

Training: (i) Practical course on Molecular Biology and course on Mycology at the Department of Plant Biology, University of Fribourg, Switzerland (2001).
(ii) Training on Teaching Methodology at GTI, BAU, Bangladesh (From 18 December,1996 to 1 January, 1997).

Publications

A. Scientific papers:

(a) On Ph. D. research:

Hamiduzzaman, M.M.; G. Jakab; L. Barnavon; J.-M. Neuhaus and B. Mauch-Mani.2005. ß-amino butyric acid-induced resistance against downy mildew in grapevine acts through the potentiation of callose formation and JA signalling. Molecular Plant-Microbe Interactions 18 (8): 819-829.

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B. Master Degree (M. S.) Thesis:

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