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Comparison of *PR1* expression in grapevine cultures after inoculation with a host- and a non-host pathogen

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Summary

***PR1* gene induction was monitored by RT-PCR in three different grapevine cultures that were inoculated with *Plasmopara viticola* as a host pathogen and *Pseudoperonospora cubensis* as a non-host pathogen. The results of the expression analysis showed that the *PR1* mRNA level in *Vitis vinifera* cv. Riesling and *Vitis riparia* cv. Gloire de Montpellier is mainly affected by the culture system. *PR1* is constitutively expressed in callus cultures. *In vitro* plants showed a low basal expression of *PR1* that is enhanced after challenge with both downy mildews in the two species tested. In greenhouse plants, *PR1* is only expressed 24 h post inoculation (hpi) but not 12 hpi. Heavily infected leaves ('oilspots') of the susceptible cv. Riesling, exhibiting well developed infection structures in the intercellular space as indicated by microscopical methods, also showed a high *PR1* expression rate three weeks post inoculation. Thus, the role of *PR1* expression in impeding the downy mildew pathogen remains equivocal. It seems that expression of *PR1* is a general stress response in some grapevine culture systems and that their use as a reference for gene expression analysis is limited.**

K e y w o r d s : Downy mildew, *Pseudoperonospora cubensis*, *Plasmopara viticola*, RT-PCR, *Vitis*.

Introduction

In search for alternative strategies in plant protection based on biological treatments, several chemicals, elicitors or other compounds have been tested in order to achieve an induction of systemic resistance and, therefore, to enhance the resistance against pathogenic microorganisms. Since a systemic acquired resistance (SAR) is accompanied by an expression of *PR1*, the accumulation of *PR1*-mRNAs and the corresponding PR1-protein after fungal or chemical challenge is often used as a marker for SAR in plants (Van Loon and van Strien 1999). Although the PR1-encoding genes of many plants have been identified and their activation after pathogen attack as well as the direct antifungal activity of the encoded protein was demonstrated, especially against oomycetic pathogens (Alexander *et al.* 1993, Niderman *et al.* 1995), the exact mechanism by which the PR1-protein acts to reduce fungal ingress is still not known. Nevertheless, as stated above, an enhanced expression of the PR1-encoding gene is considered as an enhanced resistance against phytopathogenic microorganisms and viruses. In many cases, cell suspension cultures or callus cultures were used due to an easy handling and axenic culture conditions. Moreover, they offer the possibility to test different putative inducing agents, avirulent microorganisms or pathogens. In addition, *in vitro* and greenhouse plants or even leafed single-node cuttings (Liu *et al.* 2003) were used for inoculation experiments, and it is reported, that these hosts mimic the response of field-grown plants at the cellular level, as shown in microscopical studies (Dai *et al.* 1995 a, Kortekamp and Zyprian 2003). Nevertheless, a model system may show alterations regarding the induction of genes compared to the situation occuring in nature as shown for osmotin (Monteiro *et al.* 2003) or genes of the flavonoid/stilbene pathway (Sparvoli *et al.* 1994). Therefore, the present study was conducted (1) to investigate the expression of *PR1* as a marker for resistance in different types of grapevine cultures (callus cultures, *in vitro* plants and greenhouse plants), (2) to focus on early time points, since an effective defense against biotrophic pathogens occures in grapevine within the first 24 h post inoculation (hpi), (3) to investigate the abundance of *PR1*-transcripts after an inoculation with *Plasmopara viticola* (Berk. & Curt.) Berl. & De Toni as a host-pathogen and *Pseudoperonospora cubensis* (Berk. & Curt.) Rostovzev as a non-host pathogen, and (4) to demonstrate the interaction between the two pathogens and the grapevine cultivars selected by using light and scanning electron microscopy.

Material and Methods

P l a n t c u l t u r e s a n d p a t h o g e n s : Two grapevine species, *Vitis vinifera* cv. Riesling and *Vitis riparia* cv. Gloire de Montpellier, were used. Plants were grown in the greenhouse at daylight and 20 °C or under *in vitro*-conditions as described in Blaich (1977). Callus cultures of both cultivars were derived from internodes of aseptically grown *in vitro* plants placed on LS-medium (Linsmaier and Skoog 1965) supplemented with 0.5 mg BAP l^{-1} and 0.5 mg NAA l^{-1}. For preparation of LS-media, ordinary sugar (Diamant Raffinade Zucker) was used instead of purified sucrose.

Sporangiospores of *Plasmopara viticola* were collected during summer 2003 from infested vineyards in Palatinate, Landau i.d. Pfalz, mainly from *V. vinifera* cvs Silvaner or

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Müller-Thurgau. Sporangiospore suspensions (50,000 ml^{-1}) were used as inoculum and applied as drops of 50 µl on the lower leaf surfaces. Inoculation of callus cultures was done according to DAI *et al.* (1995 b). Controls in all experiments were performed by applying the same amount of sterile water. Each experiment was conducted at least three times with two replications.

To test to what extent a non-pathogenic oomycete induces *PR1* expression in *Vitis* sp., sporangia of *Pseudoperonospora cubensis* were also used as an inoculum and applied in the same manner as described for *P. viticola*. Sporangia of *Ps. cubensis* were obtained from infected cucumber plants (*Cucumis sativus* L.) cultivated in the greenhouse of the Institute for Phytomedicine (RÖHNER *et al.* 2004).

Microscopy: Parts of the samples used for the RNA extraction were also prepared for microscopical analysis. Samples for light and scanning electron microscopy were prepared following the method described by KORTEKAMP *et al.* (1998). Cleared leaf fragments were also stained with 0.01 % (w/v) Uvitex 2B (Ciba Chemicals, Lampertheim, Germany) in 0.1 M Tris/HCl-buffer (pH 8.0) or 0.1 % (w/v) Phloxin B (Sigma, Taufkirchen, Germany) in distilled water (KORTEKAMP 2005).

Expression analysis: For gene expression analysis, leaf pieces were harvested at several time intervals after inoculation with the two pathogens. Total RNA was extracted by scaling down the experimental conditions previously described (CHANG *et al.* 1993). Plant tissue (100 mg) was ground in liquid nitrogen to fine powder using mortar and pestle. About 500 µl extraction buffer (100 mM Tris-HCl, pH 8.0, 25 mM EDTA, 2 M NaCl, 0.5 g l^{-1} spermidine, 2 % (w/v) CTAB, 2 % (w/v) PVP K 30, 2 % (v/v) β-mercaptoethanol) were added and mixed thoroughly. The lysate was extracted twice with 500 µl of chloroform:isoamylalcohol (24:1, v/v). The upper phase was transferred to a new tube and the RNA was precipitated with 0.25 volume of 10 M LiCl at 4 °C over night. The RNA was collected by a centrifugation step (4 °C, 15000 g) in a table top centrifuge. After dissolving the pellet in 500 µl 0.1 % SDS, the solution was extracted again with 500 µl chloroform:isoamylalkohol. The RNA was precipitated with 2 volumes of ethanol for two h at -20 °C, washed with 70 % cold ethanol, air dried for 20 min and resuspended in 50 µl sterile water treated with diethyl pyrocarbonate (DEPC). For RT-PCR, total RNA was incubated with one unit RNase-free DNase I per one µg RNA for 30 min at 37 °C and stopped with a chloroform:isoamylalcohol mixture (24:1, v/v). DNase-treated RNA (1 µg) was calculated using a GeneQuant II (Pharmacia Biotech, Cambridge, England) and reverse transcribed using the Revert Aid cDNA Synthesis Kit (MBI Fermentas) following the manufacturer´s instructions. RT-PCR was performed using a standard protocol with 30 cycles at 58 °C (PR1) or 65 °C (*β-tubulin*) as annealing temperature and 1 µl of diluted cDNA according to TIMMUSK and WAGNER (1999). Primer sequences were designed based on the sequence information from the NCBI database (GenBank) for PR1 (AJ003113) and *β-tubulin* (AF196485): PR1F 5'-CAT TGC ACA GAA TTA TGC TAA CC-3', PR1R 5'-CCT GGA GGA TCA TAG TTG CAA G; TubF 5'-GCA GTG AAC CTG ATC CCA TTT CC-3', TubR 5'-GCT CAC TCA CCC TCC TGA ACA TC-3'. All chemicals were purchased from Sigma (Taukirchen, Germany) and MBI Fermentas (St. Leon-Rot, Germany) except for primer pairs, which were obtained from MWG-Biotech (Ebersberg, Germany).

Results

In order to check the quality of cDNA after isolation of RNA and reverse transcription, the expression of *β-tubulin* was analysed first, and then used as an internal standard. Serial dilutions of cDNA were prepared for PCRs using gene sequence-specific primers (Fig. 1). These dilutions were employed, since PCR amplification is exponential and band intensity differences in the gel must be measured before saturation of the amplification reaction is reached. The control sequence of the *β-tubulin* gene was amplified to equal intensity when one µl of diluted cDNA was used and PCR was carried out with 30 cycles (Fig. 1).

Since *P. victicola* is an obligate biotrophic fungus, that grows within the intercellular spaces of infected leaves, the plant origin of the *PR1*-PCR product was confirmed in PCR reactions with genomic DNA of Gloire de Montpellier, Riesling, and *P. viticola*. Specific amplificates of the expected size (291 bp) were obtained from both *Vitis* cultivars tested, but never from the fungal DNA (data not shown). The specific PCR products were cloned, analysed and verified as an extracellular *PR1* (Table). Furthermore, control PCRs with native total RNA or RNase-treated RNA did not lead to the expected PCR product, indicating that the RNA was not contaminated with DNA prior reverse transcription.

In callus cultures (Fig. 2 a), *PR1* gene expression was observed in both grapevine cultivars and in all samples. Since basal expression of *PR1* occured in non-treated cells in this culture system, there was no further induction of *PR1* transcription in inoculated callus cultures as determined on the basis of the RT-PCR experiments. Also suspension cultures of cv. Riesling showed a strong basal expression of *PR1* (not shown). In *in vitro*-plants, an increased expres-

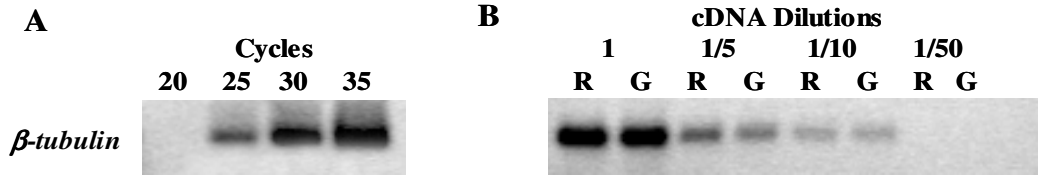

Fig. 1: Reverse transcription-PCR with different cycle numbers and cDNA dilutions. One example of an analysis performed on undiluted cDNA of non-inoculated callus cultures of Riesling is shown (**a**). Serial dilutions of this cDNA and also of cDNA from callus cultures of Gloire de Montpellier were subjected to RT-PCR (30 cycles) with β-*tubulin* specific primers as a control for equal RNA/cDNA content in the sample (**b**); R = Riesling, G = Gloire.

T a b l e

BLASTn results (GenBank, NCBI) showing homology of the PR1 sequence obtained after RT-PCR with gene specific primers

GenBank Accession no.	Homology	E-value
AJ003113	*Vitis vinifera* partial mRNA for PR1 protein	7e-168
AJ536326	*Vitis vinifera* mRNA for putative PR1 precursor	3e-68
AY298726	*Vitis vinifera* pathogenesis-related protein (PR1) mRNA	2e-08
X06930	*Nicotiana tabacum* PR1a gene for pathogenesis-related protein	9e-04
U49241	*Nicotiana glutinosa* pathogenesis-ralated protein 1	0.86

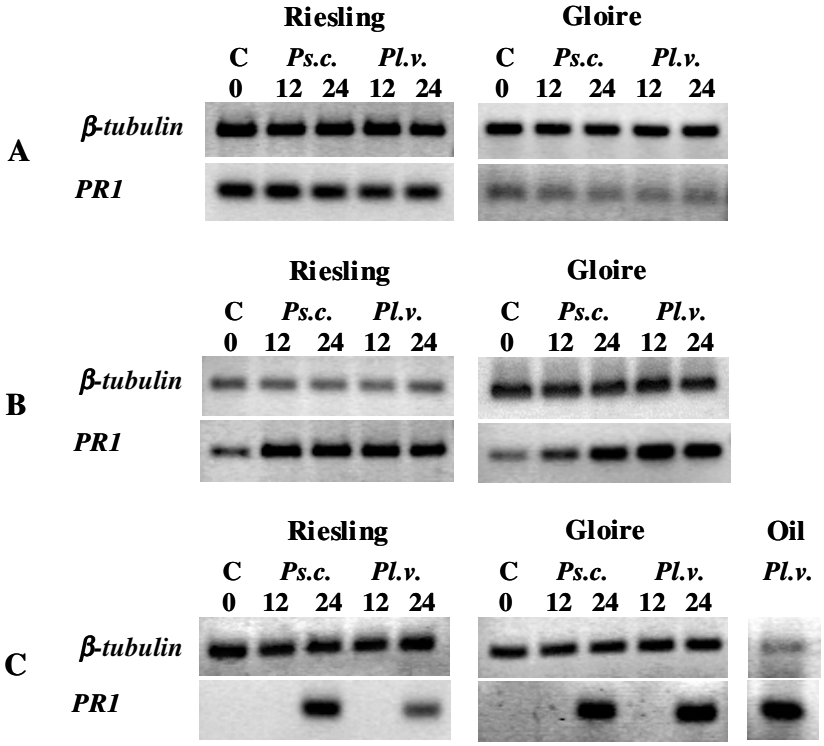

Fig. 2: Expression pattern of *β-tubulin* and *PR1* in callus cultures (**a**), *in vitro*-plants (**b**), and leaves of greenhouse plants (**c**) of Riesling and Gloire de Montpellier 12 and 24 h post inoculation. 'Oil' represents infected leaf tissue of Riesling ('oil spots') 3 weeks post inoculation that was excised and used for RNA preparation; C = non-inoculated control, *Ps.c.* = inoculated with *Pseudoperonospora cubensis*, *Pl.v.* = inoculated with *Plasmopara viticola*.

sion of *PR1* was observed in both species after inoculation with *Ps. cubensis* and *P. viticola* compared to the control and the expression pattern of *β-tubulin* (Fig. 2 b). In both grapevine species, expression of *PR1* was low in the untreated controls, but increased during the following 12 and 24 hpi, respectively. In contrast to the *in vitro* plants, there was no basal expression of *PR1* in untreated leaves of greenhouse-grown plants (Fig. 2 c). Furthermore, *PR1*-transcripts were only detectable at 24 hpi but not at 12 hpi in both species, and in Riesling the abundance seemed to be higher after inoculation with *Ps. cubensis* compared to the *P. viticola*-inoculated plants and the *β-tubulin* expression pattern. Heavily infected leaf areas ('oil spots') of Riesling showed a high level of *PR1* expression 3 weeks post inoculation. At this time, leaf samples were also investigated by different microscopical techniques. A dense net of intercellularly hyphae had developed and was clearly seen in the scanning electron (Fig. 3 a) and light microscope (Fig. 3 b), respectively. The production of haustoria as feeding organs (Fig. 3 c), sporangiophores with sporangia, and oospores (Fig. 3 d) as resting spores, that were formed later on during the infection process in the infected leaves, indicated progressive growth of *P. viticola*. Fungal growth in Riesling was only restricted in case for *Ps. cubensis*, indicating that grapevine is not a host for this pathogen. Inoculation of Riesling with *Ps. cubensis* led to a hypersensitive response (Fig. 4) which was also observed in Gloire either after inoculation with *P. viticola* or the non-host pathogen (Fig. 4).

Discussion

Light- and electron-microscopical studies revealed that zoospores of *P. viticola* could initiate an infection process

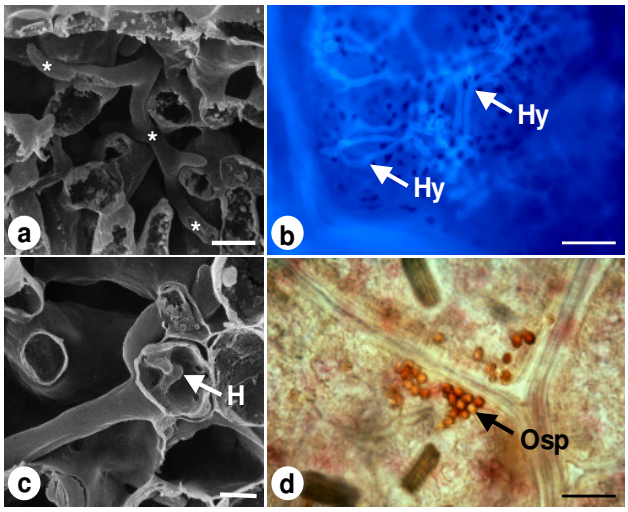

Fig. 3: Scanning electron and light microscopical investigation of *P. viticola* in infected Riesling leaves ('oil spots') 3 weeks after inoculation; (**a**) Growth of a hypha between mesophyll cells; one hypha is indicted by asterisks, bar = 10 µm; (**b**) infected leaf area with Uvitex 2B-stained mycelium; Hy = hypha, bar = 50 µm; (**c**) well developed haustoria (H) in an infected cell, bar = 5 µm; (**d**) production of oospores (Osp) occuring in the infected leaf area, Phloxin B stain, bar = 50 µm.

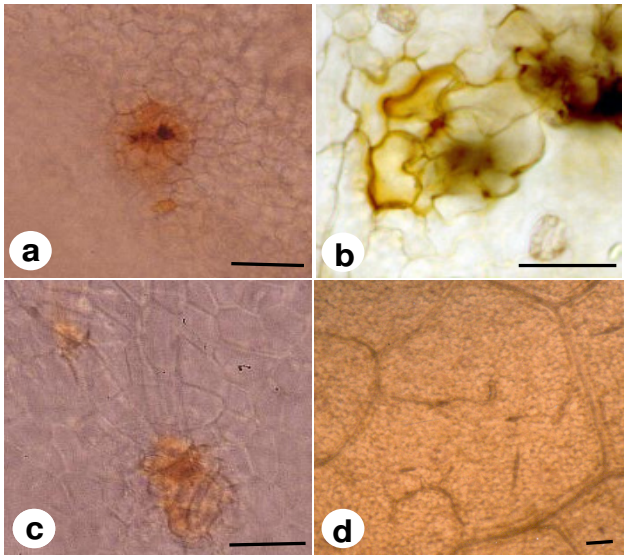

Fig. 4: Induction of a hypersensitive response (HR) in leaves of greenhouse plants of Gloire de Montpellier (**a** and **b**) and Riesling (**c** and **d**) 12 h post inoculation with the two downy mildews. The HR occurs in Gloire after inoculation with with *Ps. cubensis* (**a**) and *P. viticola* (**b**), in Riesling only after inoculation with *Ps. cubensis* (**c**) but not with *P. viticola* (**d**); bars = 30 µm.

within one to two h after inoculation via encystment in vicinity of stomata and subsequent germ tube production on both, resistant and susceptible grapevine plants. Even though zoospores of other downy mildew fungi also accumulate around stomata (KORTEKAMP 2003) and are capable infecting grapevine leaves and inducing non-host resistance (KORTEKAMP and ZYPRIAN 2003), there are only weak differences in expression rates of *PR1* in plants either treated with the host pathogen or the non-host pathogen in both, resistant and susceptible species. Nevertheless, there are differences in regard to *PR1*-expression between cell populations of different origin. An expression of *PR1* in greenhouse-grown plants was only detected in Riesling and in Gloire 24 hpi with the host and non-host pathogen which was in contrast to other culture systems such as *in vitro*-plants. In that case, the respective transcripts were only present at a basal level in non-treated controls, but were clearly enhanced at 12 hpi and 24 hpi. REUVENI (1998) reported that expression of PR-proteins such as PR2 (β-1,3-glucanase) or PR9 (peroxidase) seems to depend on leaf age. As plants are juvenalized *in vitro*, they do not show age-related resistance as observed in field- or greenhouse-grown plants and may therefore also show alterations in expression of *PR*-genes. MONTEIRO *et al.* (2003) have shown that osmotin accumulates in healthy leaves of grapevine plants cultured *in vitro*, indicating non-optimal growing conditions. Furthermore, sucrose and glucose, normal components of culture media, act as stimuli for the expression of *PR*-genes. The effects of both sugar molecules are not due to osmotic effects (THIBAUD *et al.* 2004). These stimuli may also be present in callus cultures. Since callus cultures consist of undifferentiated cell aggregates and do not represent normal leaf tissue, they may show an untypical expression pattern of stress-induced genes. This seems also to be the case for suspension cultures, where a clear basal expression could be observed in non-treated Riesling cells (not shown). In *Arabidopsis* cell cultures, THIBAUD *et al.* (2004) have shown that PR2 and PR5 (osmotin) proteins were permanently present in the culture medium and the authors suggested that the synthesis at a basal level could be due to stress generated during rotation of the cells in flasks. Although these cultures are widely used due to easy handling, their use as a reference for gene expression analysis seems to be limited. Plants grown in the greenhouse seem to represent a better source for investigations of specific gene activation. Nevertheless, expression of *PR1* may be a general response to an unspecific stimulus and is thus not only activated in response to infections against oomycetic pathogens. For other plant species it was shown that *PR1* is induced under non-pathogenic, developmentally regulated events (NAVARRE and MAYO 2004), such as flowering (LOTAN *et al.* 1989) or cytokinin fluctuation (MEMELINK *et al.* 1987), and as a consequence of expression of the *LOX* gene (ZABBAI *et al.* 2004). Expression of *PR1* in the susceptible cultivar Riesling was seen after inoculation with the non-host pathogen as well as after inoculation with the host pathogen in all culture systems tested, and there are no noteworthy differences compared to the resistant cultivar Gloire de Montpellier; thus, it seems questionable to what extent biological control in grapevine may be achieved at the level of high *PR1* expression by pre-inoculation of avirulent strains of the same pathogen, application of closely related pathogens or PR1-inducing agents. This is in agreement with MONOT *et al.* (2002) who have shown that broccoli seedlings, exhibiting an induced resistance, accumulate the PR-proteins PR2 and PR5 but not PR1, PR3 and PR9 after pre-inoculation with avirulent isolates of *Peronospora parasitica*. This indicates that PR1 does not take part in an induced resistance in the host-pathogen relationship men-

tioned above, and this may be also the case in other host-downy mildew interactions. Although mature PR1 proteins are able to inhibit the growth of oomycete pathogens, as described in the introduction, they have a very limited antifungal activity (VAN LOON and VAN STRIEN 1999). The role of grapevine *PR1* expression in impeding fungal ingress or subsequent colonization of grape tissues still remains equivocal, since *PR1* was highly expressed in heavily infected tissues of the susceptible cultivar and its induction was furthermore not correlated with a hypersensitive response. Screening strategies that are applied in order to identify inducers of SAR should include the expression of other (*PR*-)genes or the abundance of their corresponding proteins as well as an appropriate culture system.

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