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Biosynthesis of Pterostilbene in Grapevine

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A Stress-Inducible Resveratrol O-Methyltransferases Involved in the Biosynthesis of Pterostilbene in Grapevine

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ABSTRACT

Stilbenes are considered the most important phytoalexin group in grapevine (*Vitis vinifera*) and they are known to contribute to the protection against various pathogens. The main stilbenes in grapevine are resveratrol and its derivatives and, among these, pterostilbene has recently attracted much attention, due to both its antifungal and pharmacological properties. Indeed, pterostilbene is 5 to 10 times more fungitoxic than resveratrol *in vitro* and recent studies have shown that pterostilbene exhibits anti-cancer, hypolipidemic and anti-diabetic properties. A candidate gene approach was used to identify a grapevine resveratrol O-methyltransferase (ROMT) cDNA and the activity of the corresponding protein was characterized, after expression in *Escherichia coli*. Transient co-expression of ROMT and grapevine stilbene synthase in tobacco using the agroinfiltration technique resulted in the accumulation of pterostilbene in tobacco tissues. Taken together these results showed that ROMT was able to catalyse the biosynthesis of pterostilbene from resveratrol both *in vitro* and *in planta*. *ROMT* gene expression in grapevine leaves was induced by different stresses, including downy mildew (*Plasmopara viticola*) infection, UV light and $AlCl_3$ treatment.

INTRODUCTION

Stilbenes represent a small class of plant secondary metabolites derived from the phenylpropanoid pathway. In addition to their participation in both constitutive and inducible defense mechanisms in plants, several stilbenes display important pharmacological properties, and in this respect, the best-studied stilbene is resveratrol (3,5,4'-trihydroxy-trans-stilbene). Hundreds of studies have shown that resveratrol can prevent or slow the progression of a wide variety of illnesses, including cancer, and cardiovascular disease, as well as extend the lifespans of various organisms (Baur and Sinclair, 2006). However, pterostilbene, a methyl ether of resveratrol, has recently attracted much attention, as a growing number of reports describe promising pharmacological properties. Pterostilbene (3,5-dimethoxy-4'-hydroxy-trans-stilbene) was first isolated from *Pterocarpus santalinus* (red sandalwood) (Seshadri, 1972) and is one of the active constituents of *Pterocarpus marsupium*, used in traditional medicine for the treatment of diabetes. Indeed, pterostilbene was found to decrease significantly plasma glucose levels in hyperglycemic rats (Manickam et al., 1997). Pterostilbene has also been reported to have hypolipidemic properties comparable to clinically used fibrate lipid-lowering drugs (Rimando et al., 2005). The targets of fibrate drugs are members of the peroxisome proliferator activated receptors (PPARs) group of transcription factors, which play a major role in regulating lipoprotein metabolism (Kersten, 2008). Activation of PPARα in mice and humans reduces hepatic triglyceride production and promotes plasma triglyceride clearance, leading to a reduction in plasma triglyceride levels. Pterostilbene was found to be very effective as a lipid/lipoprotein lowering agent in hypercholesterolemic hamsters, probably due to the fact that, like fibrate drugs, pterostilbene was shown to act as a PPARα agonist (Rimando et al., 2005). In addition, pterostilbene exhibits antioxidant and anti-cancer properties similar to those of resveratrol (Jang et al., 1997). Pterostilbene was found to be very effective in preventing carcinogen-induced preneoplastic lesions in a mouse mammary organ culture model (Rimando et al., 2002) and it showed preventive activity against colon carcinogenesis in rats (Suh et al., 2007).

Pterostilbene accumulates constitutively in various organs from a small number of plant species, including wood from *P. santalinus* and *Vaccinium* berries (Seshadri,

1972, Rimando et al. 2004). Pterostilbene has also been identified as a phytoalexin in grapevine (*Vitis vinifera*). Together with resveratrol and its derivatives piceid and viniferins, pterostilbene accumulates in grapevine leaves infected by *Plasmopara viticola* (Langcake et al., 1979), and low amounts of pterostilbene are also produced during the preparation of grapevine protoplasts (Commun et al. 2003). This compound was also found in healthy grape berries as a constitutive stilbene (Pezet & Pont, 1988). Like resveratrol, pterostilbene was shown to possess antifungal activity against various grapevine pathogens (Langcake et al., 1979). Indeed, pterostilbene was 5 to 10 times more effective than resveratrol in inhibiting the germination of conidia of *Botrytris cinerea* and sporangia of *P. viticola* (Jeandet et al., 2002). Despite its high antifungal activity and its promising pharmacological properties, the biosynthetic pathway that produces pterostilbene has not yet been characterised. There is not even any direct evidence that pterostilbene is derived from resveratrol by methylation and earlier attempts to characterize a putative resveratrol O-methyltransferase that could catalyse the direct conversion of resveratrol into pterostilbene were unsuccessful (Jeandet et al., 2002).

Several O-methyltransferases (OMTs) active with substrates structurally analogous to resveratrol have been characterized. For example, in Scots pine (*Pinus sylvestris*), pinosylvin (3,5-dihydroxystilbene) can be methylated by a pinosylvin O-methyltransferase (PMT) to pinosylvin 3-O-methyl ether, following ozone or fungal elicitor treatment (Chiron et al., 2000a). In roses, orcinol O-methyltransferases (OOMTs) have been shown to catalyse the methylation of orcinol to yield the volatile phenolic ether 3,5-dimethoxytoluene, a major compound of rose scent (Lavid et al., 2002; Scalliet et al., 2002). Starting from these results, we used a candidate gene approach to identify a previously uncharacterized resveratrol O-methyltransferase (ROMT) involved in the biosynthesis of pterostilbene in grapevine. We show that *ROMT* gene expression in grapevine leaves is induced by fungal infection, UV light and $AlCl_3$ treatment.

RESULTS

Biosynthesis of Pterostilbene in Grapevine Leaves Infected with *Plasmopara viticola*

In order to characterize a putative ROMT from grapevine, the variety Cabernet Sauvignon was chosen, as it was shown previously to accumulate pterostilbene upon fungal infection (Langcake et al., 1979). Indeed, inoculation of Cabernet Sauvignon leaves with *Plasmopara viticola* resulted in the accumulation of various stilbenes, including trans-resveratrol, trans- and cis-piceid, viniferins and pterostilbene (Fig. 1). UV treatment resulted in a massive accumulation of stilbenes, to tenfold the levels observed following fungal infection. However, when we specifically quantified pterostilbene, this molecule did not accumulate to as high a relative level in the UV treated leaves as in the *P. viticola*-infected leaves. Stilbene quantifications showed leaf to leaf variations, probably due to differences in leaf developmental stages, as maturity has been shown to influence *P. viticola* infection (Liu et al., 2003). A number of plant OMTs have been characterized using biochemical approaches (He and Dixon, 1996; Wang et al., 1997; Chiron et al., 2000a; Dudareva et al., 2000; Wu et al., 2004). These strategies typically involved enzyme purification and sequencing of peptides derived from the purified protein. However, this kind of approach can be used only if sufficient enzyme activity is detected in plant extracts. When incubated with resveratrol and S-adenosyl-L-[methyl-^{14}C] methionine ([^{14}C]SAM), both *P. viticola*-infected and UV-treated Cabernet Sauvignon leaf extracts failed to exhibit significant ROMT activity (data not shown). This may be due to a low abundance or a poor extractability of the ROMT enzyme. Therefore, a candidate gene approach was chosen to search for grapevine *ROMT*.

Isolation of a Candidate Resveratrol O-methyltransferase cDNA from Grapevine

To search for candidate resveratrol-OMTs, we first looked for OMTs that have been shown to be active with substrates that are structurally analogous to resveratrol. Such enzymes include PMT from *Pinus sylvestris* and OOMTs from roses. However, PMT was shown to methylate pinosylvin on one hydroxyl group only to yield pinosylvin 3-O-methyl ether (Chiron et al., 2000a). Conversely, OOMTs can methylate both hydroxyl groups of orcinol at position 3 and 5, to yield the dimethylated molecule 3,5-dimethoxytoluene (Fig. 2) (Lavid et al., 2002; Scalliet et al., 2002). In previous studies, we characterized OOMTs from *Rosa chinensis* cv Old Blush (Scalliet et al., 2002; 2006), and preliminary experiments showed that resveratrol could be methylated by purified recombinant OOMT1 from Old Blush.

Therefore, we searched grapevine public EST collections for OOMT homologues. Blast searches identified several ESTs with homology to rose OOMTs, and their sequences were used to design oligonucleotides to PCR amplifiy the corresponding full-length coding sequence. Assuming that ROMT is expressed in *P. viticola*-infected tissues, leaves from Cabernet Sauvignon were infected by immersion in a suspension of *P. viticola* sporangia (2.10^4 mL^{-1}) and total leaf RNAs were prepared 24 hours post infection. RT-PCR allowed the amplification of a single 1,3 kb DNA fragment (data not shown) that was cloned into pGEM T-easy and 20 different pGEM clones were sequenced. All clones were very similar, with minor differences probably corresponding to allelic forms. The most represented clone (15 out of 20) was selected as a candidate ROMT. This putative ROMT cDNA from Cabernet Sauvignon shared 74% nucleotide sequence identity with rose OOMT1. The predicted amino acid sequence of the candidate ROMT shared 69.5 % and 30% identity with rose OOMT1 and PMT from *P. sylvestris*, respectively (Fig. 3A). Phylogenetic analysis of selected members of the plant small molecule OMT gene family (Noel et al., 2003) showed that PMT and ROMT belong to distinct clades corresponding to different OMT subfamilies (Fig. 3B).

Characterization of Recombinant Resveratrol O-methyltransferase

The ROMT coding sequence was then cloned into the Gateway compatible entry vector pDNOR207, and then transferred into the destination vector pHNGWA (Busso et al., 2005) to express the corresponding protein in *E. coli*. ROMT was expressed as a NusA fusion protein, purified and cleaved on the resin with thrombin to yield purified recombinant ROMT enzyme. In order to characterize its putative resveratrol O-methyltransferase activity, purified recombinant ROMT was incubated with resveratrol, resveratrol monomethyl ether (3-methoxy-4',5-dihydroxy-trans-stilbene) and pterostilbene, in the presence of [^{14}C]SAM. Thin layer chromatography (TLC) analysis of reaction products showed that, after a short incubation of ROMT with resveratrol and [^{14}C]SAM, two products were produced that co-migrated with resveratrol monomethyl ether and pterostilbene standards, respectively. ROMT was also able to methylate resveratrol monomethyl ether to yield pterostilbene. Conversely, no methylation product of pterostilbene was detected (Fig. 4A). GC-MS analysis following a longer incubation of ROMT with resveratrol and a twofold molar amount of SAM identified pterostilbene as the major reaction product (Fig. 4B).

ROMT exhibited equivalent Km values for resveratrol and resveratrol monomethyl ether (12 µM and 14 µM, respectively), however, resveratrol monomethyl ether was the preferred substrate, with Kcat and specific activity values being significantly higher than for resveratrol. The activity of ROMT was then tested *in vitro* with a number of potential substrates. ROMT exhibited a remarkable preference for resveratrol and resveratrol monomethyl ether over all compounds tested. Indeed, the specific activities of ROMT with orcinol, eugenol, or caffeic acid were a few percent of its specific activity with its preferred substrate resveratrol monomethyl ether (Table 1).

Characterization of Resveratrol O-methyltransferase Activity *in Planta*

Recombinant ROMT exhibited resveratrol-OMT activity *in vitro*, catalysing the biosynthesis of pterostilbene from resveratrol, in the presence of SAM. However, predicting the physiological substrate of OMTs from *in vitro* data only is not trivial (Schröder et al., 2002). In a previous work, we used *Agrobacterium*-mediated transient transformation of *Nicotiana benthamiana* to characterize rose OOMT activity (Scalliet et al., 2006). Therefore, we chose the same approach to investigate ROMT activity *in planta*. For *in planta* expression, the ROMT coding sequence was transferred in the Gateway compatible plant transformation vector pB2GW7 (Karimi et al., 2002), to yield the pB2GW7-ROMT construct. In order to provide ROMT with resveratrol substrate, ROMT was co-expressed with the grapevine stilbene synthase cDNA VST1 (Melchior and Kindl, 1991). Transgenic expression of VST1 in various plant species including tobacco, tomato, papaya and grapevine has been shown to result in resveratrol and resveratrol derivatives accumulation in transformed plants (Hain et al., 1990; Thomzik et al., 1997; Hipskind and Paiva, 2000; Coutos-Thévenot et al., 2001). As a control, tobacco leaves were infiltrated with *Agobacterium* harbouring a 35S-GFP construct (Haseloff et al., 1997). No stilbenes were detected in extracts from leaves expressing GFP (Fig. 5A). However, significant amounts of resveratrol and piceid accumulated when stilbene synthase was expressed (Fig. 5B) and the co-expression of stilbene synthase and ROMT resulted in a marked decrease in the resveratrol and piceid peaks, together with a strong accumulation of pterostilbene (Fig. 5C). The pterostibene peak was collected and its identity was confirmed by GC-MS analysis (data not shown). Together, these results showed that

ROMT from Cabernet Sauvignon possessed resveratrol-OMT activity catalysing the biosynthesis of pterostilbene from resveratrol both *in vitro* and *in planta*.

Analysis of ROMT Gene Expression

Both biotic and abiotic stresses (UV exposure, $AlCl_3$ treatment) have been shown to induce the accumulation of stilbenes and the expression of genes involved in stilbene biosynthesis in grapevine (Fritzmeier and Kindl, 1981; Adrian et al., 1996; Douillet-Breuil et al., 1999, Kortekamp, 2006). Therefore, we analysed *ROMT* genes expression in grapevine leaf discs submitted to abiotic stresses and fungal infection. Equal amounts of total RNA from control (t=0), mock-infected, *P. viticola*-infected, UV-treated and $AlCl_3$-treated grapevine leaf discs were used for semi-quantitative RT-PCR analysis of *ROMT*, *PAL* and *STS* expression. Total RNAs were prepared 0, 6, 24 and 48 hours after treatment and actin was used as a control (Fig. 6). *PAL* and *STS* expression was induced 6 hours post-treatment compared to the control (t=0), in *P. viticola*-infected and in UV- and $AlCl_3$-treated leaf discs, and transcript levels remained high 48h post-treatment. *PAL* and *STS* were also induced in mock-infected discs, presumably as a result of the wounding response following disc cutting. Indeed, wound induction of *PAL* and *STS* gene expression has been shown to occur in different plant species (Lawton and Lamb, 1987; Chiron et al., 2000b). Like *PAL* and *STS, ROMT* gene expression was induced in *P. viticola*-infected and in UV- and $AlCl_3$-treated leaf discs, reaching a maximum 24 hours post-treatment. However, *ROMT* gene expression was not induced in mock-infected discs, indicating that this gene does not respond to the stress induced by this experimental procedure, that include wounding stress due to disc cutting. These results were confirmed in an independent experiment, using real-time quantitative RT-PCR (Fig. 7). *ROMT* expression was transiently induced in response to *P. viticola* infection, with a maximum 24 hours post-infection.

DISCUSSION

Characterization of a Novel Resveratrol O-methyltransferase From Grapevine

Although pinosylvin closely resembles resveratrol, PMT is only able to methylate pinosylvin on one hydroxyl group, to yield pinosylvin 3-O-methyl ether (Chiron et al.,

2000a). Therefore, we hypothesized that the putative ROMT enzyme was more likely to resemble rose OOMTs, which catalyse the biosynthesis of the dimethylated molecule 3,5-dimethoxytoluene (Fig.2) (Lavid et al., 2002; Scalliet et al., 2002). Identification of OOMT homologues among grapevine EST collections allowed the cloning of a candidate ROMT cDNA from Cabernet Sauvignon. The predicted amino acid sequence of this putative ROMT shared 69.5 % and 30% identity with rose OOMT1 and *P. sylvestris* PMT, respectively (Fig. 3A). Phylogenetic analysis of selected members of the plant OMT gene family showed that PMT and ROMT belong to different subfamilies. Indeed, PMT is related to the caffeic acid-OMT (COMT) gene family, whereas ROMT belongs to a distinct clade, which includes rose OOMT, chavicol and eugenol-OMT from basil (Gang et al., 2002) (Fig. 3B). Stilbenes occur naturally in a small number of unrelated plant species and phylogenetic analysis of the stilbene synthase (*STS*) and chalcone synthase (*CHS*) gene family showed that *STS* genes have evolved from *CHS* genes several times independently in the course of evolution (Tropf et al., 1994). Consistent with this repeated evolution of *STS* genes, the stilbene methylation enzymes PMT and ROMT also evolved independently in Scots pine and grapevine, respectively, from different OMT ancestors. Unlike PMT, which was shown to be active with a broad range of substrates (Chiron et al., 2000a), ROMT exhibited a remarkable specificity for resveratrol and resveratrol monomethyl ether (Table 1). Determination of ROMT kinetic parameters showed that Km values for resveratrol and resveratrol monomethyl ether were similar (12 µM and 14 µM, respectively), however, ROMT exhibited a significantly higher specific activity with resveratrol monomethyl ether as a substrate than with resveratrol (Table 1). This may explain why, when ROMT was incubated with resveratrol in the presence of SAM, the resveratrol monomethyl ether intermediate accumulated only if the incubation time was short (Fig 4A). When longer incubation times were used, the only product observed was pterostilbene and no accumulation of the monomethyl intermediate was detected (Fig 4B). Pterostilbene accumulation following co-expression of *ROMT* and *STS* in tobacco (Fig. 5C), confirmed that ROMT alone was sufficient to catalyse both resveratrol methylation steps to yield pterostilbene. In contrast, the biosynthesis of DMT from orcinol in roses (Fig. 2) involves two different OOMT1 and OOMT2 enzymes, which operate sequentially to catalyse the first and the second methylation step of orcinol, respectively (Lavid et al., 2002). The evolution of *OOMT1* from duplicated *OOMT2*

gene most probably corresponds to an optimization of DMT biosynthesis in Chinese roses (Scalliet et al., 2008). A Blast search of the genome of the highly homozygous PN40024 grapevine cultivar (Jaillon et al., 2007) identified a small family of 4 putative ROMT genes located on chromosome 12 (accessions CAO69896, CAO69893, CAO69817 and CAO69813), all of which were very similar to ROMT from Cabernet Sauvignon. However, none of these genes was identical to the *ROMT* gene characterized in this work, which probably represents an allele of the CAO69896 gene of PN40024. Indeed, the protein deduced from the CAO69896 gene differed in only 3 amino acid positions from ROMT from Cabernet Sauvignon. Additional work will be needed to characterize the function of the other members of this gene family.

ROMT Gene Expression is Induced by Biotic and Abiotic Stresses

UV light, aluminium chloride ($AlCl_3$) treatments and fungal infection have been shown to induce the accumulation of stilbenes in grapevine leaves (Fritzmeier and Kindl, 1981; Adrian et al., 1996; Douillet-Breuil et al., 1999). However, in grapevine tissues, pterostilbene usually accumulates in low amounts compared to other resveratrol derivatives (Langcake et al., 1979; Pezet and Pont, 1988). Inoculation of Cabernet Sauvignon leaves with *P. viticola* resulted in an accumulation of pterostilbene corresponding to about 5% total stilbene content (Fig. 1). Stilbene accumulation following UV treatment was much higher, reaching tenfold the abundance detected after fungal infection. However, pterostilbene accumulation in UV-treated leaves was relatively lower, corresponding to only 1.5% of total stilbenes. This could be explained by a limiting ROMT activity in Cabernet Sauvignon leaves. Although *ROMT* gene expression was induced following both *P. viticola* infection and UV exposure, ROMT enzyme activity may not have been sufficient to produce high amounts of pterostilbene. Trans- and cis-piceid accounted for 30% to 50% of total stilbenes in *P. viticola*-infected or UV-treated leaves (Fig.1), indicating that glucosyltransferases may have competed with ROMT for the resveratrol substrate (Hall and De Luca, 2007). Furthermore, stilbene quantification following elicitation of grape cell suspensions showed that most of the resveratrol was secreted into the culture medium (Aziz et al., 2003). Therefore, resveratrol methylation may also be impaired by secretion mechanisms that lower its availability to ROMT.

In Scots pine, *PMT* gene transcription was shown to be induced by various stresses including ozone treatment, fungal infection and wounding (Chiron et al., 2000b). Semi-quantitative RT-PCR analysis of *ROMT* expression showed that, like *PAL* and *STS* genes*, ROMT* expression was induced in *P. viticola*-infected and in UV- and $AlCl_3$-treated leaf discs. *ROMT* transcript accumulated transiently, reaching a maximum 24 hours post-treatment, and decreased markedly afterwards. These results were confirmed using real-time quantitative RT-PCR (Fig. 7). Quantification of *ROMT* transcripts in response to *P. viticola* infection showed that, although they accumulated following infection, their absolute number remained low in comparison to *PAL* or *STS* transcripts. This relatively low level of *ROMT* transcript accumulation, together with the weak activity of the corresponding enzymes in grapevine extracts, may explain why earlier attempts to characterize ROMT were unsuccessful (Jeandet et al., 2002).

Pterostilbene Accumulates as the Major Stilbene Following In Planta Co-expression of *STS* and *ROMT* Genes

Although STS enzymes occur naturally in only a few plants species, the substrates necessary to synthesize stilbenes are present in all higher plants. In consequence, the ability to synthesize stilbenes can be conferred to many plant species by expressing an *STS* gene (Hain et al., 1990; Hipskind and Paiva, 2000; Yu et al., 2006). In this work, we used tobacco leaves capable of stilbene biosynthesis due to the transient expression of the grapevine stilbene synthase VST1 cDNA to characterize the activity of ROMT *in planta*. Unlike grapevine, where pterostilbene accumulates to relatively low amounts, pterostilbene was the major stilbene in tobacco leaves co-expressing *STS* and *ROMT* (Fig. 5). Given the antibiotic properties of stilbenes, *STS* genes have been used as targets for transgenic crop enhancement. *STS* genes from peanut and grape have been expressed in tobacco and alfalfa, respectively, and in both cases, enhanced resistance to pathogenic fungi was conferred to the transgenic host plants (Hain et al., 1990; Hipskind & Paiva, 2000). Due to the higher fungitoxicity of pterostilbene compared to resveratrol (Pezet & Pont, 1990; Pezet et al., 2004), the combination of *STS* and *ROMT* genes in transgenic plants may lead to improved resistance characteristics. In particular, such transgenic plants will allow a detailed investigation of the fungitoxic properties of

pterostilbene *in vivo*, in order to complete the data obtained from *in vitro* studies (Langcake et al., 1979; Pezet & Pont, 1990). Recent transcript profiling experiments have been aimed at identifying the molecular pathways affected by pterostilbene exposure in yeast (Pan et al., 2008). These included lipid and methionine metabolism and further characterization of cellular targets of pterostilbene may provide new insights into the molecular basis of its fungitoxicity.

In conclusion, we characterized a grapevine ROMT catalyzing the efficient biosynthesis of pterostilbene from resveratrol, both *in vitro* and *in* planta. The anti-cancer, hypolipidemic and anti-diabetic properties of pterostilbene have recently attracted much interest (Rimando et al. 2005). In particular, the high hypolipidemic activity of pterostilbene may provide alternative treatments of dyslipidemia in the future. Thus, the identification of ROMT constitutes a major step in understanding the biosynthesis of a plant stilbene with promising pharmacological properties.

Materials and Methods

Chemicals and Radiochemicals

Trans-resveratrol and *trans*-pterostilbene were from Sigma-Aldrich (Saint Quentin Fallavier, France). *Trans*-piceid, *trans*-δ-viniferin and *trans*-ε-viniferin standards were kindly provided by R. Pezet (Changins, Switzerland). Cis forms of stilbenes were obtained by photoisomerization under UV light of *trans*-stilbene standard solutions at 5 μg.mL^{-1}. S-adenosyl-L-[methyl-^{14}C] methionine (55 mCi/mmol) ([^{14}C]SAM) was from GE Healthcare-Amersham Biosciences. Resveratrol monomethyl ether (3-methoxy-5,4'-dihydroxy-trans-stilbene) was obtained from a partial chemical methylation of resveratrol. 100 mg resveratrol were incubated with equimolar amounts of iodomethane and K_2CO_3 in aceton (8 mL final volume), for 3 hours at 50°C. Resveratrol monomethyl ether (RME) was purified using preparative thin layer chromatography and HPLC, and characterized by gas chromatography coupled to mass spectrometry (GC-MS). RME characteristics corresponded to published data (λ_{max} 307 nm, 320 nm in 50% acetonitrile, ion at m/z 242) (Katsuyama et al., 2007; Mikstacka et al., 2007). HPLC grade solvents (acetonitrile, methanol) were from Merck (Darmstadt, Germany) and used in combination with sterilized water from Aguettant (Lyon, France). All other chemicals and reagents were from Sigma (St. Quentin Fallavier, France).

Plant Material and Leaf Sample Preparation

Grapevine plantlets (*V. vinifera* cv. Cabernet Sauvignon) were obtained from cuttings and grown on potting soil in a greenhouse at a temperature of 22°C and 19°C (day and night, respectively), with a photoperiod of 16 h of light (supplemental light provided by sodium lamp illumination), until they developed 12-14 fully expanded leaves. The sixth leaf, counted from the apex was detached, rinsed with sterile demineralized water and leaf discs (14 mm diameter) were punched. For each sample, leaves from 4 different plantes were used and 4 leaf discs were pooled, each coming from a different plant, in order to represent the same individuals in every time points.

For aluminium chloride treatment, leaf discs were placed (abaxial face up) in Petri dishes on filter paper soaked with 5 ml of a freshly prepared 1% aluminium

chloride solution. For UV treatment, leaf discs were placed the abaxial face up in Petri dishes on wet filter paper and exposed for 6 min to UV light (90 µW cm^{-2}) from a UV-C tube (Osram, 30 W, 254 nm).

Plasmopara viticola isolate was harvested on *V. vinifera* cv. Chardonnay infected leaves in the experimental vineyard plot at INRA-Colmar in 2006 and maintained on grapevine seedlings (*V. vinifera* cv. Muscat ottonel). Leaf discs were infected by immersion in a suspension of *P. viticola* sporangia in sterile water (2.10^4 mL^{-1}) and then transferred on humid filter paper in sealed Petri dishes. Mock-inoculated leaf discs were immersed in sterile water. All leaf discs were kept at 21°C under 16/8 h photoperiod (200 µmol m^{-2} s^{-1}). Leaf discs were collected at 0, 6, 24 and 48 h after treatment, immediately frozen in liquid nitrogen and conserved at -80°C until RNA extraction.

Stilbene Analyses

Stilbene extractions and HPLC-DAD analyses were performed as described previously (Poutaraud et al. 2007). Gas chromatography-mass spectrometry (GC-MS) analyses were performed as described previously (Mikstacka et al., 2007).

Cloning of ROMT cDNA. ROMT coding region was amplified by RT-PCR using the upstream primer 5'-ATGGATTTGGCAAACGCTGTGATATCAGCTGA-3' and the downstream primer 5'-TCAAGGATAAACCTCAATGAGGGACCTCAAACC-3'. Primers were designed based on ESTs CF202077 and CF209780. Reverse transcription was performed as indicated below. PCR amplification was carried out for 30 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 30 s and extension at 72°C for 1 min with a final extension of 5 min, in a GeneAmp PCR system 9700 cycler (Perkin Elmer, NY, USA), using *Ex Taq* DNA polymerase (Takara Bio Inc., Shiga, Japan). Amplified DNA fragments were cloned into pGEM T-easy (Promega, Madison, WI, USA) and the inserts sequenced.

Phylogenetic Analysis. OMT sequences were obtained from GenBank. Nucleic acid and protein sequences were aligned using ClustalW (Thompson *et al*., 1994). Phylo_win program (Galtier *et al*., 1996) was used to construct phylogenetic trees, using the neighbor-joining method, with 500 bootstrap replicates.

Characterization of Recombinant ROMT. ROMT cDNA was amplified by PCR using the upstream primer 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTGGTTCCGCGTGGATCCATGGATTTGGCAAACGCTGTG-3' and the downstream primer 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTTCATCAAGGATAAACCTCAATGAG-3', and cloned into pDONR207 Gateway compatible vector (Invitrogen, Carlsbad, CA). ROMT cDNA was sequenced to verify that no mutation had been introduced, and subsequently transferred into the pHNGWA destination vector (accession number EU680842, Busso et al., 2005). ROMT enzyme was expressed as His-tagged NusA-fusion protein. Recombinant ROMT was purified using TALON® metal affinity resin (Clontech) and characterized after cleavage of the NusA moity using thrombin (GE Healthcare-Amersham). Purified recombinant ROMT was incubated in a final volume of 25 µl with 35 µM [^{14}C]SAM and 200 µM to 1 mM of stilbene substrates in 0.1 M Tris, pH 7.5, containing 20% glycerol (v/v), 5 mM $MgCl_2$ and 14 mM 2-mercaptoethanol. Ranges of stilbene substrate concentrations of between 5 µM and 1 mM were used for K_m determination, and the incorporated radioactivity was measured by liquid scintillation. K_m and V_{max} values were calculated from Lineweaver-Burk plots. Reaction products were analysed by TLC on silica gel (Merck, Darmstadt, Germany) with dichloromethane/aceton (40/3, v/v) as the solvent, using a Molecular Imager FX phosphorimager (Bio Rad, Hercules, CA). Enzyme reaction products were identified by co-migrating with standards.

Transient Expression in Tobacco

For Agrobacterium-mediated transient expression, ROMT cDNA was transferred into the GATEWAY-compatible binary vector pB2GW7 (Karimi et al., 2002). Grapevine stilbene synthase was expressed in tobacco using a 35S:VST1 construct described previously (Santos-Rosa et al., 2008) and m-GFP5-HDEL was used as a control (Haseloff *et al*., 1997). All constructs were introduced into *Agrobacterium tumefaciens* strain C58 (pMP90) by electroporation. *Nicotiana benthamiana* leaves were infiltrated with *A. tumefaciens* cultures (OD_{600} 0.1 to 0.3) according to Batoko *et al.* (2000). Disks were punched from tobacco leaves 48h after *Agrobacterium* infiltration and analysed for stilbene content.

RNA isolation and semiquantitative RT-PCR

Total RNAs were isolated using RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions and quantified using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Waltham, MA). Residual genomic DNA was removed by performing on-column DNase I digestion with the RNase-Free DNase Set (Qiagen). One microgram of total RNAs was used as template for reverse transcription, using the RevertAid^{TM} M-MuLV reverse transcriptase (Fermentas), with 0.5 µg of oligo(dT)$_{18}$, for 1 h at 42°C. PCR amplifications were performed on 5 µL of the 10x diluted cDNA solution using *Taq* DNA Polymerase from Promega, with 28 cycles of 94°C for 15 s, 50°C for 30 s, and 72°C for 30 s. Primers are described below. All PCR products were separated onto a 2% agarose gel stained with EtBr and image processing was carried out with a Bio-Rad GelDoc apparatus (Bio-Rad), bands analyzed with Quantity One software, version 4.4.1 (Bio-Rad).

Real-time quantitative RT-PCR

Transcript levels were determined by real-time one step RT-PCR using i-Cycler iQ^{TM} thermal Cycler (Bio-Rad) and QuantiTect® SYBR® Green RT-PCR kit (Qiagen). Total RNAs were adjusted to 2.5 ng/µL before use. RT-PCR reactions were carried out in triplicate in 96-well plates (25 µL per well) in a buffer containing 1x QuantiTect SYBR Green RT-PCR Master Mix (including HotStarTaq® DNA Polymerase, dNTPs, SYBR Green dye), 0.25 µL of QuantiTect RT Mix (containing Omniscript® and Sensiscript® Reverse Transcriptases), 10 nM fluorescein (used as dynamic well factor), 500 nM forward and reverse primers and 12.5 ng of total RNAs. One step RT-PCR reactions were performed under the following conditions: 50°C for 30 min (reverse transcription), 95°C for 15 min (RT degradation and *Taq* DNA Polymerase activation), followed by 40 cycles of denaturation at 94°C for 15 s, annealing at 60°C for 30 s, extention at 72°C for 30 s, and data acquisition at 77°C for 15 s. Identical conditions were used for all transcripts, except for grapevine *PAL* (annealing at 58°C, acquisition at 76°C) and *P. viticola* actin (acquisition at 81°C). Transcript levels were calculated using the standard-curve method and normalised using grapevine actin gene as internal control. Results presented are means of duplicate data of one representative experiment. The following primers were used fror RT-PCR amplifications: 5'-GTGCCAATTTATGAAGGTTATGC-3' and 5'-CCCTCTCAGTTAGAATCTTCATCAG-3' for grapevine actin (accession No.

AF369525), 5'-GGGATTTCACTGTTGCATATACTC-3' and 5'-CATAGAATAGAAAGCGCGAGG-3' for *PAL* (CF511227), 5'-AAAGAAACTCGAAGCAACGAGG-3' and 5'-TGGCCCTCTCCCCCTTAA-3' for STS (X76892), 5'-TGCCTCTAGGCTCCTTCTAA-3' and 5'-TTTGAAACCAAGCACTCAGA-3' for ROMT (FM178870). Actin from *P. viticola* was amplified using the primers 5'GTTCGAGACGTTCAACGTGC and 5'CATGATGGTCTGGAACGTGC. To design the primers, actin sequence from the Oomycetes *Phytophthora infestans*, *Pythium splendens* and *Achlya bisexualis* were aligned together with actin from *V. vinifera*. Primers targeted sequences conserved in all three Oomycetes but divergent in grapevine. Absence of amplification in healthy grapevine samples confirmed the specificity of the primers.

Accession Number

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession number FM178870.

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FIGURE LEGENDS

Fig. 1. Stilbene contents in control, *P. viticola*-infected and UV-treated Cabernet Sauvignon leaves. Stilbenes were analysed with HPLC-DAD, 72 h post-treatment. Note that for better readability, the stilbene content scale is different in UV-treated leaves. Standard errors were calculated based on three replicates.

Fig. 2. Biosynthesis of 3,5-dimethoxytoluene in *Rosa* and proposed biosynthesis of pterostilbene in *Vitis*

Fig. 3. Comparison of *Vitis* ROMT with other OMTs. A. The predicted amino acid sequence of *Vitis* ROMT was aligned with *R. chinensis* cv. Old Blush OOMT1 and *P. sylvestris* PMT with Clustal W. Residues shaded in grey indicate identical amino acids. B. Phylogenetic tree of selected OMT cDNA sequences: *V. vinifera* ROMT (Vv ROMT, accession number FM178870), *V. vinifera* caffeic acid-OMT (Vv COMT, AF239740), *R. chinensis* OOMT1 (Rc OOMT1, AJ439741), *Ocimum basilicum* chavicol-OMT (Ob CVOMT, AF435007), *O. basilicum* eugenol-OMT (Ob EOMT, AF435008), *Clarkia breweri* (iso)eugenol-OMT (Cb IEMT, U86760), *C. breweri* caffeic acid-OMT (Cb COMT, AF006009), *Nicotiana tabacum* caffeic acid-OMT (Nt COMT, AF484252) and *P. sylvestris* pinosylvin-OMT (PsPMT, Chiron et al., 2000). The numbers beside the branches represent bootstrap values based on 500 replicates.

Fig. 4. Analysis of ROMT Reaction Products. A. TLC analysis of reaction products produced following incubation of recombinant ROMT with 200 µM resveratrol (1), 200 µM resveratrol monomethyl ether (2) and 200 µM pterostilbene (3) in the presence of

S-adenosyl-L-[methyl-^{14}C] methionine (50 µM). Reactions were carried out in a total volume of 25 µL with 200 ng of purified protein and were allowed to proceed for 15 min. The positions of the origin (O) and the reaction products resveratrol monomethyl ether (RME) and pterostilbene (P) are indicated. B. Gas chromatography-mass spectrometry (GC-MS) analysis of ROMT reaction products. Recombinant ROMT was incubated with resveratrol (500 µM) in the presence of S-adenosyl-L-methionine (1 mM). Peak 1, pterostilbene; peak 2, resveratrol. A total ion chromatogram is shown and mass spectra of the peaks matched those of the corresponding authentic standards. The reaction was carried out in a total volume of 200 µL with 2 µg of purified protein and was allowed to proceed for 60 min.

Figure 5. Characterization of ROMT activity *in planta* using *Agrobacterium*-mediated transient transformation. Tobacco leaf sectors (150 mg fresh weight) expressing GFP (A), grapevine stilbene synthase (B), or co-expressing stilbene synthase and ROMT (C) were excised 48h after Agrobacterium-mediated transformation. Stilbene content was analysed using HPLC-DAD. Peak 1, piceid; peak 2, resveratrol; peak 3, pterostilbene. Identity of peak 3 was confirmed by GC-MS analysis (data not shown).

Fig. 6. RT-PCR analysis of *ROMT* expression in grapevine leaf discs submitted to different stresses. Sets of leaf discs were immersed in sterile ultrapure water (mock) or infected by immersion in a suspension of *P. viticola* sporangia (2.10^4 mL^{-1}) in sterile ultrapure water. UV-C irradiation (λ=254 nm, 90 µW cm^{-2}) was applied for 7 min. $AlCl_3$ solution (1 %, w/v) was applied directly on the discs. Leaf discs were collected at t=0 (control), 6, 24 and 48 hours after treatments and frozen before RNA extraction. Selected genes are actin, *ROMT*, phenylalanine ammonia-lyase (*PAL*) and stilbene synthase (*STS*). M, DNA molecular weight marker. The data shown are representative of three independent experiments.

Fig. 7. Quantitative RT-PCR analysis of ROMT expression in grapevine leaf discs infected by *P. viticola.* Transcript accumulation *ROMT* (accession No. FM178870), stilbene synthase (*STS*, accession No. X76892), phenylalanine ammonia lyase (*PAL*,

accession No. CF511227) genes were monitored in mock-infected leaf discs (open squares) and *P. viticola*-infected leaf discs (closed squares). Pathogen development was evaluated by monitoring *P. viticola* actin transcript accumulation (*P. viticola* actin). Analyses were performed by real time quantitative RT-PCR. Transcript levels of grapevine genes are expressed as relative values normalized to the transcript level of actin gene, used as an internal reference (accession No. AF369525). Absolute copy number of mRNA for each target gene in the t=0 control sample was 0 (*OMT*), 24.10^3 (*STS*), and 571.10^3 (*PAL*) molecules/µg of plant total RNAs. Results are means of duplicate experiments, bars indicate +/- standard errors.

Substrate	Km (µM)	Kcat.10^{-3} (s^{-1})	Kcat/Km ($M^{-1}.s^{-1}$)	Specific activity ($pkat.mg^{-1}$)
resveratrol	12 (4)	62 (3)	5160 (254)	786 (30)
resveratrol monomethyl ether	14 (3)	86 (9)	6142 (325)	1097 (108)
pterostilbene	n.d.	n.d.	n.d.	5 (4)
orcinol	n.d.	n.d.	n.d.	10 (6)
caffeic acid	n.d.	n.d.	n.d.	58 (7)
eugenol	n.d.	n.d.	n.d.	75 (8)

Table 1. Kinetic parameters of ROMT with potential substates. Data are expressed as the means of triplicate assays and standard errors are indicated in brackets. N.d.: not determined.

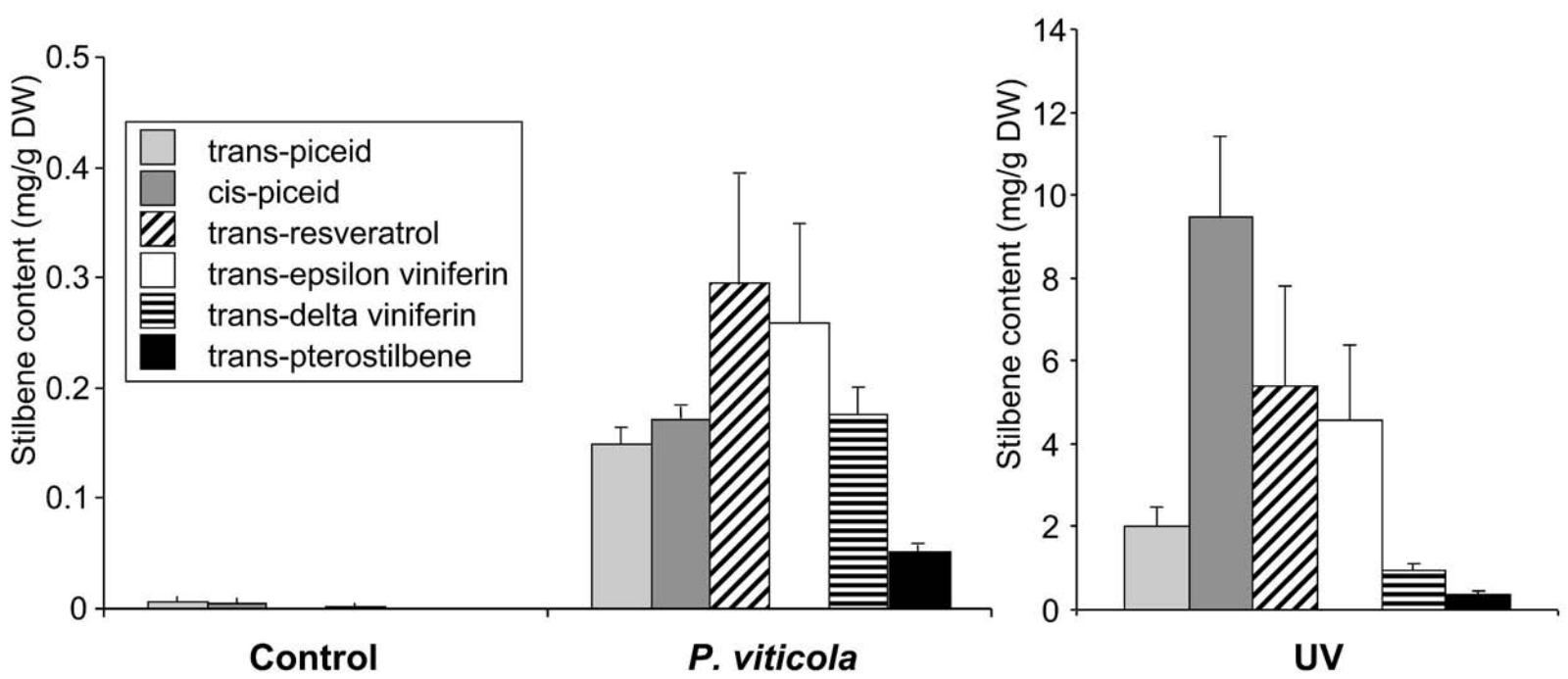

Fig. 1. Stilbene contents in control, *P. viticola*-infected and UV-treated Cabernet Sauvignon leaves. Stilbenes were analysed with HPLC-DAD, 72 h post-treatment. Note that for better readability, the stilbene content scale is different in UV-treated leaves. Standard errors were calculated based on three replicates.

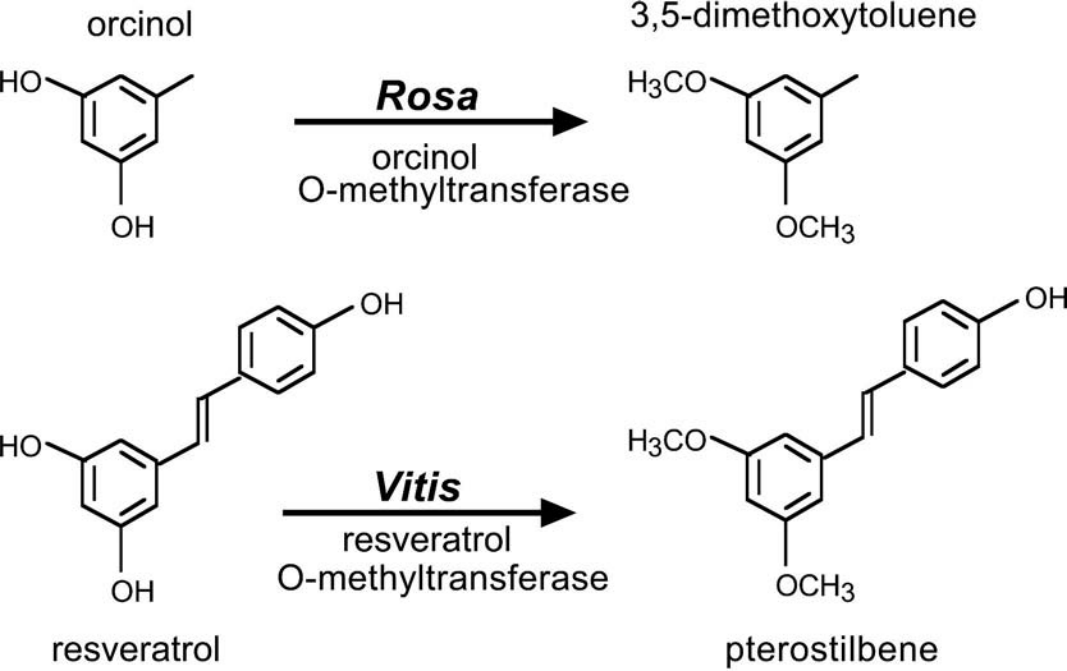

Fig. 2. Biosynthesis of 3,5-dimethoxytoluene in *Rosa* and proposed biosynthesis of pterostilbene in *Vitis*

A

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- - - - - - - - - - - M D L A N G V I S A E L L H A Q A H V W N  Vv ROMT
M E R L N S F R H L N Q K W S N G E H S N E L L H A Q A H I W N  Rc OOMT1
- - - - - - - - - - M G S A S E S S E M N A K I V N E D E W L L G  Ps PMT

H I F N - - F I K S M S L K C A I Q L G I P D I I H N H G K P M  Vv ROMT
H I F S - - F I N S M S L K S A I Q L G I P D I I N K H G Y P M  Rc OOMT1
M E L G N F S C V P M A M K A A I E L D V L Q I I A N A G N G V  Ps PMT

T L P E L V A K L P V H P K R S Q C V Y R L M R I L V H S G F L  Vv ROMT
T L S E L T S A L P I H P T K S H S V Y R L M R I L V H S G F F  Rc OOMT1
Q L S P R Q I V A H S H S V L S C S V T - - - - - - - - - - - T  Ps PMT

A A Q R V Q Q G K E E E G Y V L T D A S R L L L M D - D S L S I  Vv ROMT
A K K K L S K - T D E E G Y T L T D A S Q L L L K D - H P L S L  Rc OOMT1
D E N - - - - G K A E R L Y G L T P L C K Y L V K N K D G V S L  Ps PMT

R P L V L A M L D P I L T K P W H Y L S A W F Q N D D P T P F H  Vv ROMT
T P Y L T A M L D P V L T N P W N Y L S T W F Q N D D P T P F D  Rc OOMT1
A P L V L M N Q D K V L M E S W Y Y L K D A V L D G S - Q P F T  Ps PMT

T A H E R S F W D Y A G H E P Q L N N S F N E A M A S D A R L L  Vv ROMT
T A H G M T F W D Y G N H Q P S I A H L F N D A M A S D A R L V  Rc OOMT1
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T S V L L K E G Q G V F A G L N S L V D V G G G T G K V A K A I  Vv ROMT
T S V I I N D C K G V F E G L E S L V D V G G G T G T L A K A I  Rc OOMT1
M N K I L D T Y E G - F K E V Q E L V D V G G G V G S T L N L I  Ps PMT

A N A F P H L N C T V L D L P H V V A G L Q G S K N L N Y F A G  Vv ROMT
A D A F P H I E C T V L D L P H V V A D L Q G S K N L K Y T G G  Rc OOMT1
V S K Y P H I S G I N F D M P H V V A D A P H Y P A V K H V G G  Ps PMT

D M F E A I P P A D A I L L K W I L H D W S D E E C V K I L K R  Vv ROMT
D M F E A V P P A D T V L L K W I L H D W S D E E C I K I L E R  Rc OOMT1
D M F D S V P S G Q A I F M K W I L H D W S D D H C L R L L K N  Ps PMT

C R E A I P S K E N G G K V I I I D M I M M K N Q G D Y K S T E  Vv ROMT
S R V A I T G K E K K G K V I I I D M M M E N Q K G D E E S I E  Rc OOMT1
C H K A L P E K - - - G K V I V V D T I L P V A A E T S P Y A R  Ps PMT

T Q L F F D M T M M I F A P G - R E R D E N E W E K L F L D A G  Vv ROMT
T Q L F F D M L M M A L V G G - K E R N E K E W A K L F T D A G  Rc OOMT1
Q G F H I D L L M L A Y N P G G K E R T E Q E F R D L A K E V G  Ps PMT

F S H Y K I T P I L G L R - - S L I E V Y P                    Vv ROMT
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B

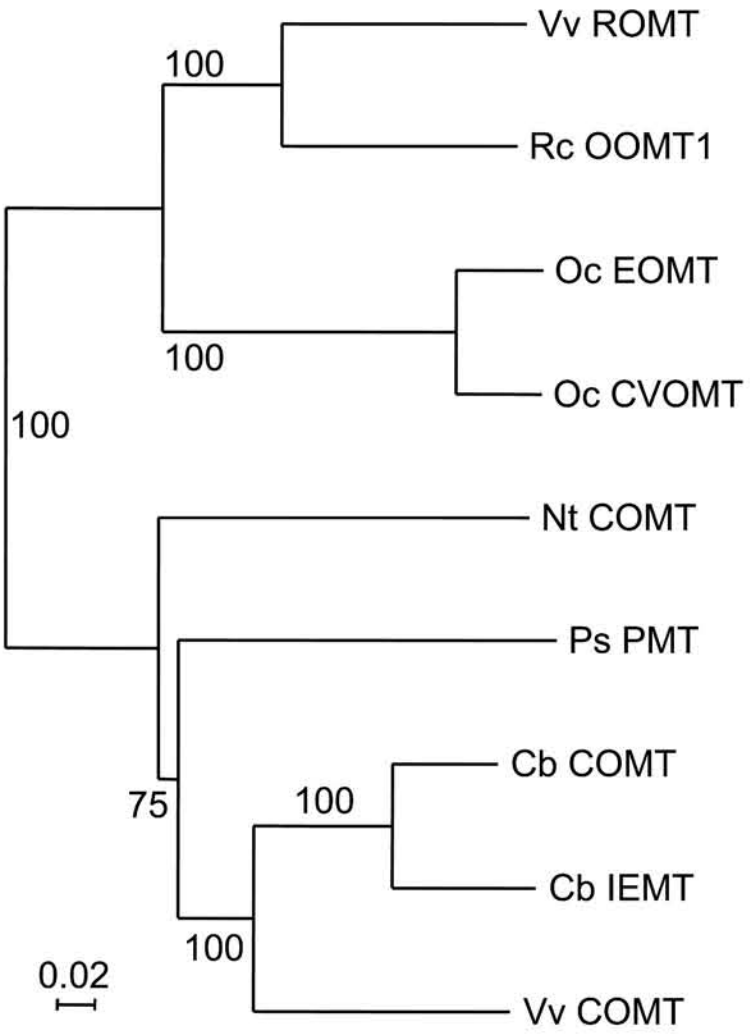

Fig. 3. Comparison of *Vitis* ROMT with other OMTs. A. The predicted amino acid sequence of *Vitis* ROMT was aligned with *R. chinensis* cv. Old Blush OOMT1 and *P. sylvestris* PMT with Clustal W. Residues shaded in grey indicate identical amino acids. B. Phylogenetic tree of selected OMT cDNA sequences: *V. vinifera* ROMT (Vv ROMT, accession number FM178870), *V. vinifera* caffeic acid-OMT (Vv COMT, AF239740), *R. chinensis* OOMT1 (Rc OOMT1, AJ439741), *Ocimum basilicum* chavicol-OMT (Ob CVOMT, AF435007), *O. basilicum* eugenol-OMT (Ob EOMT, AF435008), *Clarkia breweri* (iso)eugenol-OMT (Cb IEMT, U86760), *C. breweri* caffeic acid-OMT (Cb COMT, AF006009), *Nicotiana tabacum* caffeic acid-OMT (Nt COMT, AF484252) and *P. sylvestris* pinoresinol-OMT (Ps PMT, Chiron et al., 2000). The numbers beside the branches represent bootstrap values based on 500 replicates.

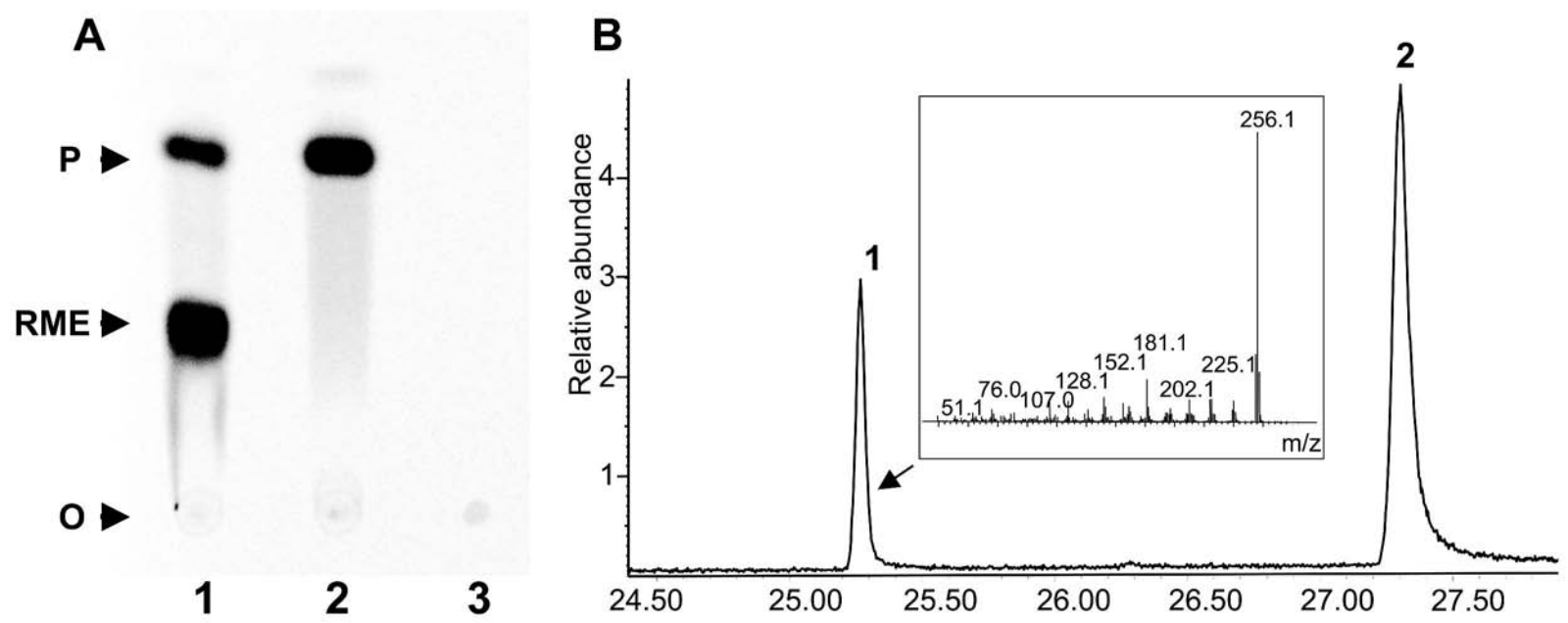

Fig. 4. Analysis of ROMT Reaction Products. A. TLC analysis of reaction products produced following incubation of recombinant ROMT with 200 μM resveratrol (1), 200 μM resveratrol monomethyl ether (2) and 200 μM pterostilbene (3) in the presence of S-adenosyl-L-[methyl-14C] methionine (50 μM). Reactions were carried out in a total volume of 25 μL with 200 ng of purified protein and were allowed to proceed for 15 min. The positions of the origin (O) and the reaction products resveratrol monomethyl ether (RME) and pterostilbene (P) are indicated. B. Gas chromatography-mass spectrometry (GC-MS) analysis of ROMT reaction products. Recombinant ROMT was incubated with resveratrol (500 μM) in the presence of S-adenosyl-L-methionine (1 mM). Peak 1, pterostilbene; peak 2, resveratrol. A total ion chromatogram is shown and mass spectra of the peaks matched those of the corresponding authentic standards. The reaction was carried out in a total volume of 200 μL with 2 μg of purified protein and was allowed to proceed for 60 min.

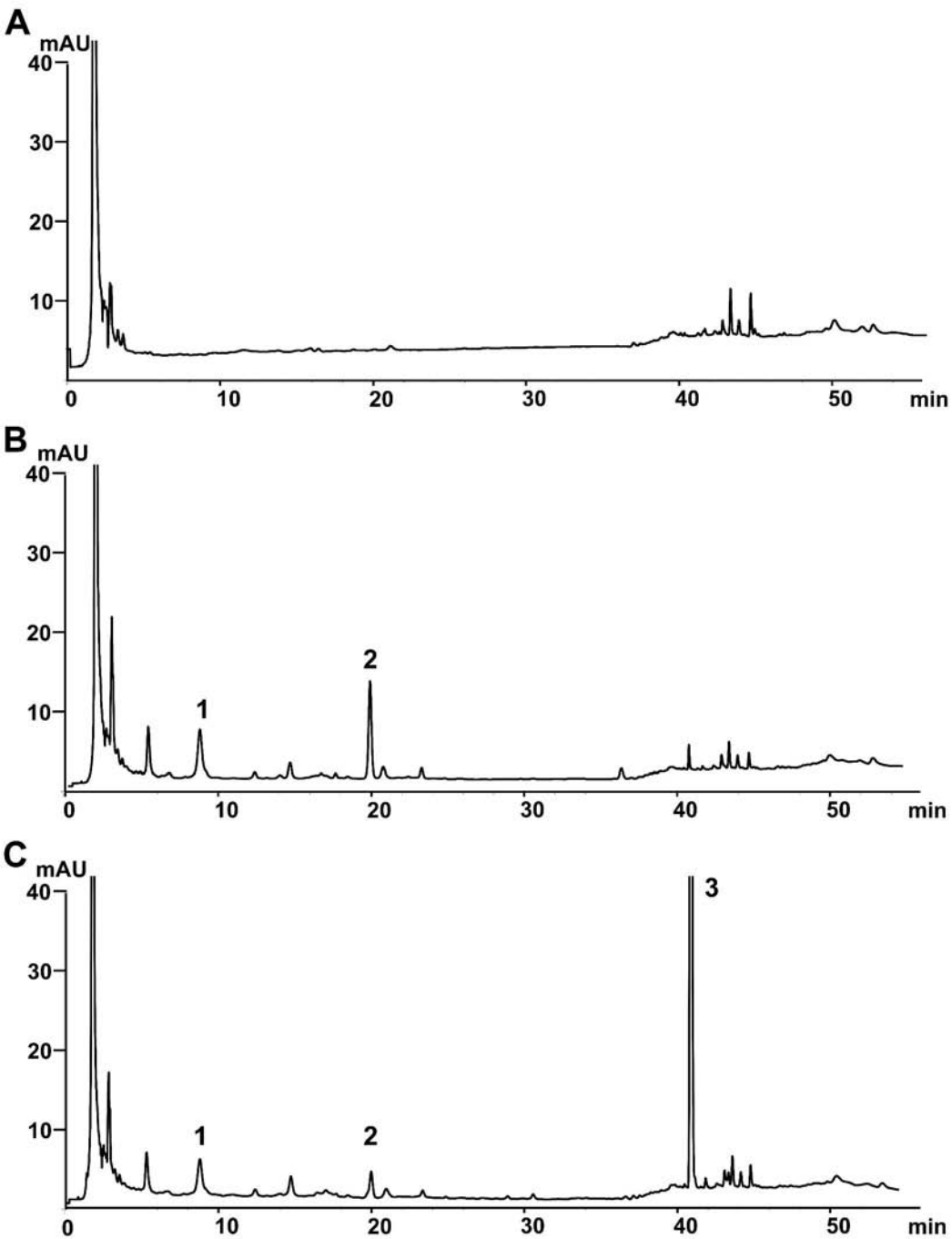

Figure 5. Characterization of ROMT activity *in planta* using *Agrobacterium*-mediated transient transformation. Tobacco leaf sectors (150 mg fresh weight) expressing GFP (A), grapevine stilbene synthase (B), or co-expressing stilbene synthase and ROMT (C) were excised 48h after Agrobacterium-mediated transformation. Stilbene content was analysed using HPLC-DAD. Peak 1, piceid; peak 2, resveratrol; peak 3, pterostilbene. Identity of peak 3 was confirmed by GC-MS analysis (data not shown).

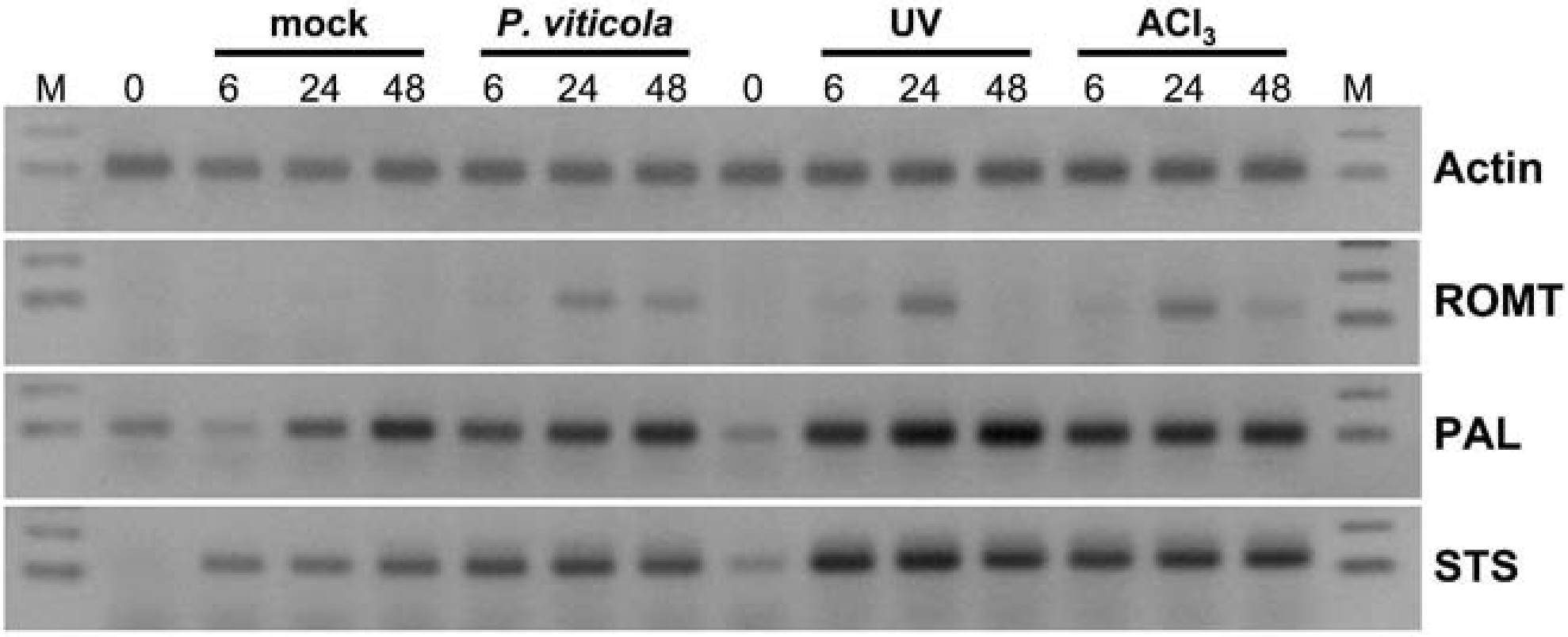

Fig. 6. RT-PCR analysis of *ROMT* expression in grapevine leaf discs submitted to different stresses. Sets of leaf discs were immersed in sterile ultrapure water (mock) or infected by immersion in a suspension of *P. viticola* sporangia (2.104 mL-1) in sterile ultrapure water. UV-C irradiation (λ=254 nm, 90 µW cm-2) was applied for 7 min. AlCl3 solution (1 %, w/v) was applied directly on the discs. Leaf discs were collected at t=0 (control), 6, 24 and 48 hours after treatments and frozen before RNA extraction. Selected genes are actin, *ROMT*, phenylalanine ammonia-lyase (*PAL*) and stilbene synthase (*STS*). M, DNA molecular weight marker. The data shown are representative of three independent experiments.

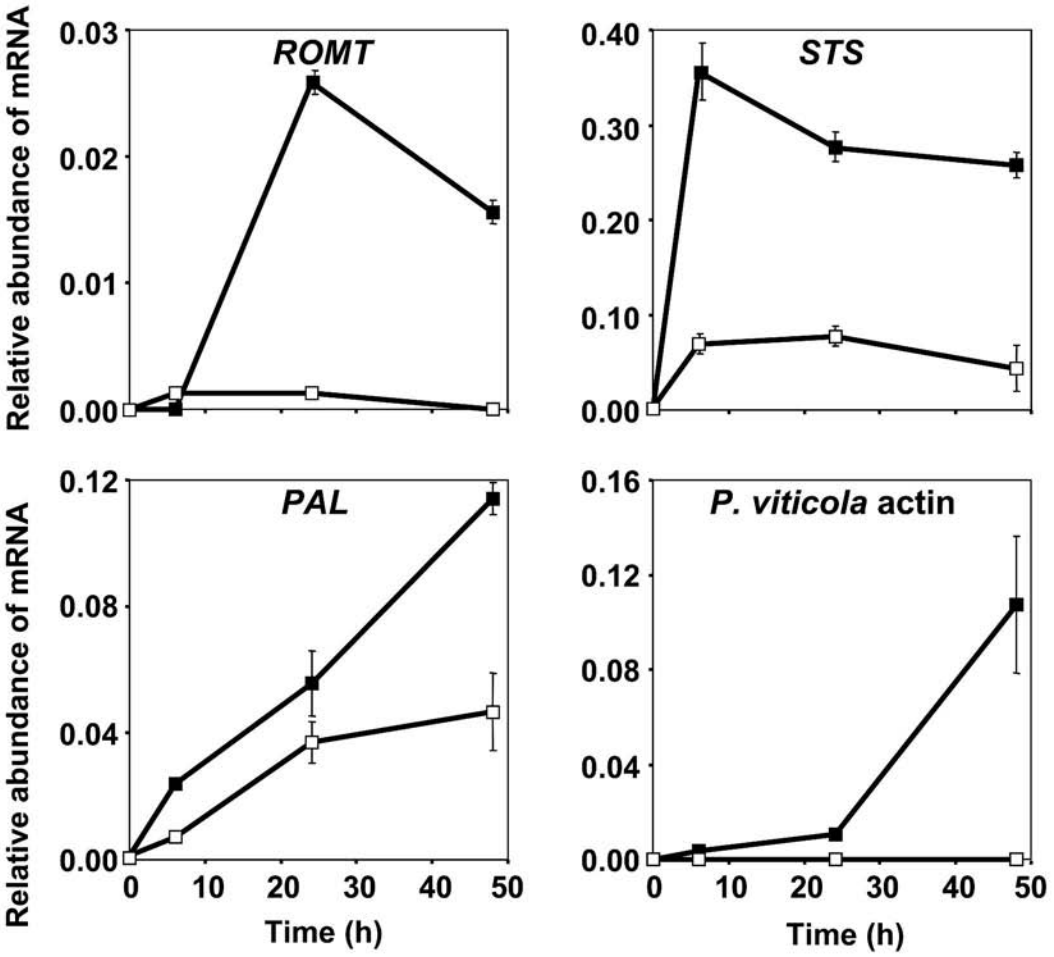

Fig. 7. Quantitative RT-PCR analysis of ROMT expression in grapevine leaf discs infected by *P. viticola*. Transcript accumulation *ROMT* (accession No. FM178870), stilbene synthase (*STS*, accession No. X76892), phenylalanine ammonia lyase (*PAL*, accession No. CF511227) genes were monitored in mock-infected leaf discs (open squares) and *P. viticola*-infected leaf discs (closed squares). Pathogen development was evaluated by monitoring *P. viticola* actin transcript accumulation (*P. viticola* actin). Analyses were performed by real time quantitative RT-PCR. Transcript levels of grapevine genes are expressed as relative values normalized to the transcript level of actin gene, used as an internal reference (accession No. AF369525). Absolute copy number of mRNA for each target gene in the t=0 control sample was 0 (*OMT*), 24.103 (*STS*), and 571.103 (*PAL*) molecules/µg of plant total RNAs. Results are means of duplicate experiments, bars indicate +/- standard errors.