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

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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 both to 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 anticancer, hypolipidemic, and antidiabetic 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 coexpression of ROMT and grapevine stilbene synthase in tobacco (Nicotiana benthamiana) using the agroinfiltration technique resulted in the accumulation of pterostilbene in tobacco tissues. Taken together, these results showed that ROMT was able to catalyze 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, ultraviolet light, and AlCl₃ treatment.

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 red sandalwood (Pterocarpus santalinus; Seshadri, 1972) and is one of the active constituents of Pterocarpus marsupium, which is used in traditional medicine for the treatment of diabetes. Indeed, pterostilbene was found to decrease significantly plasma Glc 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 receptor (PPAR) 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 anticancer 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 and 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 *Botrytis 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 characterized. 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 (ROMT) that could catalyze 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 (*Rosa* spp.), orcinol O-methyltransferases (OOMTs) have been shown to catalyze 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 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 AlCl₃ treatment.

**RESULTS**

**Biosynthesis of Pterostilbene in Grapevine Leaves Infected with *P. viticola***

To characterize a putative ROMT from grapevine, the variety Cabernet Sauvignon was chosen because it was shown previously to accumulate pterostilbene upon fungal infection (Langcake et al., 1979). Indeed, inoculation of Cabernet Sauvignon leaves with *P. viticola* resulted in the accumulation of various stilbenes, including trans-resveratrol, trans- and cis-piceid, viniferins, and pterostilbene (Fig. 1). UV treatment resulted in massive accumulation of stilbenes to 10-fold 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 because 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-3-[methyl-¹⁴C] Met ([¹⁴C]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 low abundance or poor extractability of the ROMT enzyme. Therefore, a candidate gene approach was chosen to search for grapevine ROMT.

**Isolation of a Candidate ROMT 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 Scots pine 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 positions 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 Chinese rose (Rosa chinensis) cv Old Blush (Scalliet et al., 2002, 2006), and preliminary experiments showed that resveratrol could be methylated by purified recombinant OOMT1 from cv Old Blush. Therefore, we searched grapevine public EST collections for OOMT homologs. BLAST searches identified several ESTs with homology to rose OOMTs and their sequences were used to design oligonucleotides to PCR amplify the corresponding coding sequences. Assuming that OOMT 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 h postinfection. Reverse transcription (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 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 Scots pine, 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 ROMT

The ROMT coding sequence was then cloned into the Gateway-compatible entry vector pPDNOR207, and then transferred into the destination vector pHNGWA (Busso et al., 2005) to express the corresponding protein in Escherichia coli. ROMT was expressed as a NusA fusion protein, purified and cleaved on the resin with thrombin to yield purified recombinant ROMT enzyme. To characterize its putative ROMT activity, purified recombinant ROMT was incubated with resveratrol, resveratrol monomethyl ether (RME; 3-methoxy-4′,5-dihydroxy-trans-stilbene), and pterostilbene in the presence of [14C]SAM. Thin-layer chromatography (TLC) analysis of reaction products showed that, after a short incubation of ROMT with resveratrol and [14C]SAM, two products were produced that comigrated with RME and pterostilbene standards, respectively. ROMT was also able to methylate RME to yield pterostilbene. Conversely, no methylation product of pterostilbene was detected (Fig. 4A). Gas chromatography (GC)-mass spectrometry (MS) analysis following a longer incubation of ROMT with resveratrol and a 2-fold molar amount of SAM identified pterostilbene as the major reaction product (Fig. 4B).

ROMT exhibited equivalent K_m values for resveratrol and RME (12 and 14 μM, respectively); however, RME was the preferred substrate, with K_m 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 RME 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 RME (Table I).

Characterization of ROMT Activity in Planta

Recombinant ROMT exhibited ROMT activity in vitro, catalyzing 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. To provide ROMT with resveratrol substrate, ROMT was coexpressed with the grapevine stilbene synthase (STS) cDNA VST1 (Melchior and Kindl, 1991). Transgenic expression of VST1 in various plant species, including tobacco (Nicotiana spp.), tomato (Solanum lycopersicum), papaya (Carica papaya), and grapevine, has been shown to result in resveratrol and resveratrol derivative accumulation in transformed plants (Hain et al., 1990; Thomzik et al., 1997; Hipskind and Paiva, 2000; Coutos-Thevenot et al., 2001). As a control, tobacco leaves were infiltrated with Agrobacterium harboring 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

Figure 2. Biosynthesis of 3,5-dimethoxytoluene in Rosa and proposed biosynthesis of pterostilbene in grapevine.
accumulated when STS was expressed (Fig. 5B) and the coexpression of STS and ROMT resulted in a marked decrease in the resveratrol and piceid peaks, together with strong accumulation of pterostilbene (Fig. 5C). The pterostilbene 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 ROMT activity catalyzing the biosynthesis of pterostilbene from resveratrol both in vitro and in planta.

Analysis of ROMT Gene Expression

Both biotic and abiotic stresses (UV exposure, AlCl₃ 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 analyzed ROMT gene 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 AlCl₃-treated grapevine leaf discs were used for semi-quantitative RT-PCR analysis of ROMT, PAL (for Phe ammonia-lyase), and STS expression. Total RNAs were prepared 0, 6, 24, and 48 h posttreatment and actin was used as a control (Fig. 6). PAL and STS expression was induced 6 h posttreatment compared to the control (t = 0) in P. viticola-infected and in UV- and AlCl₃-treated leaf discs and transcript levels remained high 48 h posttreatment. 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 AlCl₃-treated leaf discs, reaching a maximum 24 h posttreatment. 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, which includes 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 h postinfection.

DISCUSSION

Characterization of a Novel ROMT from Grapevine

Although pinosylvin closely resembles resveratrol, PMT is only able to methylate pinosylvin on one...
The analysis of the number of unrelated plant species and phylogenetic
et al., 2002; Fig. 3B). Stilbenes occur naturally in a small
Ocimum basilicum
and eugenol OMT from basil (Chiron et al., 2000a), ROMT1 and
OMT2 enzymes, which operate sequentially to cat-
alyzed coexpression of ROMT and STS in tobacco (Fig. 5C) confirmed that ROMT alone was sufficient to catalyze both resveratrol methylation steps to yield pterostilbene. In contrast, the biosynthesis of DMT from orcinol in roses (Fig. 2) involves two different OOMT1 and
OMT2 enzymes, which operate sequentially to cat-
yze the first and the second methylation step of
incubated with resveratrol (500 μM) in the presence of [14C]SAM (1 μM). 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.

Table 1. Kinetic parameters of ROMT with potential substrates

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m$ (μM)</th>
<th>$k_{cat}/K_m$ (s⁻¹)</th>
<th>Specific Activity (pkat mg⁻¹)</th>
</tr>
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<tbody>
<tr>
<td>Resveratrol</td>
<td>12 (4)</td>
<td>62 (3)</td>
<td>786 (30)</td>
</tr>
<tr>
<td>RME</td>
<td>14 (3)</td>
<td>86 (9)</td>
<td>1,097 (108)</td>
</tr>
<tr>
<td>Pterostilbene</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Ornicol</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
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<tr>
<td>Eugenol</td>
<td>n.d.</td>
<td>n.d.</td>
<td>75 (8)</td>
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Data are expressed as the means of triplicate assays and s.s. are indicated in parentheses. n.d., Not determined.
However, none of these genes was identical to the ROMT gene characterized in this article, which probably represents an allele of the CAO69896 gene of PN40024. Indeed, the protein deduced from the CAO69896 gene differed in only three 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, AlCl₃ 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 10-fold 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). Semiquantitative 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 AlCl₃-treated leaf discs. ROMT tran-
Pterostilbene Accumulates as the Major Stilbene following in Planta Coexpression of STS and ROMT Genes

Although STS enzymes occur naturally in only a few plant 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 STS 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 coexpressing 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 (Arachis hypogaea) and grape have been expressed in tobacco and alfalfa (Medicago sativa), respectively, and in both cases, enhanced resistance to pathogenic fungi was conferred to the transgenic host plants (Hain et al., 1990; Hipskind and Paiva, 2000). Due to the higher fungitoxicity of pterostilbene compared to resveratrol (Pezet and 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 detailed investigation of the fungitoxic properties of pterostilbene in vivo to complete the data obtained from in vitro studies (Langcake et al., 1979; Pezet and Pont, 1990). Recent transcript-profiling experiments have been aimed at identifying the molecular pathways affected by pterostilbene exposure in yeast (Saccharomyces cerevisiae; Pan et al., 2008). These included lipid and Met 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 anticancer, hypolipidemic, and antidiabetic 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...
MATERIALS AND METHODS

Chemicals and Radiochemicals

trans-Resveratrol and trans-pterostilbene were from Sigma-Aldrich, trans-Piceid, trans-6-viniferin, and trans-6-viniferin standards were kindly provided by R. Pezet (Changins, Switzerland). cis forms of stilbenes were obtained by photosomizerization under UV light of trans-stilbene standard solutions at 5 μg mL⁻¹. [14C]SAM (55 mCi/mmol) was from GE Healthcare-Amersham Biosciences. RME (3-methoxy-5,4'-dihydroxy-trans-stilbene) was obtained from a chemical method of resveratrol. One hundred milligrams of resveratrol were incubated with equimolar amounts of iodo-
methane and K₂CO₃ in acetone (8 mL final volume) for 3 h at 50°C. RME was purified using preparative TLC and HPLC, and characterized by GC-MS. RME characteristics corresponded to published data (Kmax 307 nm, 320 nm in 50% acetonitrile, ion at m/z 242; Katsuyma et al., 2007; Mikstacka et al., 2007). HPLC-grade solvents (acetonitrile, methanol) were from Merck and used in combination with sterilized water from Aguettant. All other chemicals and reagents were from Sigma.

Plant Material and Leaf Sample Preparation

Grapevine (Vitis vinifera ‘Cabernet Sauvignon’) plants 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 to 14 fully expanded leaves. The sixth leaf, counted from the base of the shoot, was isolated by immersion in a suspension of Plasmodiophora betae isolate (2×10⁶ mL⁻¹) and then transferred on humid filter paper in sealed petri dishes (Osmar; 30 W, 254 nm).

Plasmodiophora betae isolate was harvested on grapevine cv Chardonnay-infected leaves in the experimental vineyard plot at INRA-Colmar in 2006 and maintained on grapevine (cv Muscat ottonelle) seedlings. Leaf discs were infected by immersion in a suspension of P. betae sporangia in sterile water (2×10⁶ 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). 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'-ATGAGTTGGCAGAACGCGTGATACAGCTGGA-3' and the downstream primer 5'-TCAAGGATAAAACCCGAGGGCGCCTAACCACCC-3'. Primers were designed based on ESTs CF202077 and CF209780. RT 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. PCR products were cloned into pDONR207 Gateway-compatible vector (Invitrogen). Amplicons were sequenced to verify that no mutation had been introduced, and subsequently transferred into the pHN65A vector destination (accession no. EU680842; Busso et al., 2005). ROMY 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 moiety using thrombin (GE Healthcare-Amersham). Purified recombinant ROMMT was incubated in a final volume of 25 μL with 35 μCi [14C]SAM and 200 μM to 1 μM of stilbene substrates in 0.1 Tris, pH 7.5, 20% glycerol (v/v), 5 mM MgCl₂, and 14 μM imidazole. Reaction products were analyzed by TLC on silica gel (Merck) with dichloromethane:acetone (40/3 v/v) as the solvent, using a Molecular Imager FX phosphorimager (Bio-Rad). Enzyme reaction products were identified by comigrating with standards.

Phylogenetic Analysis

OMT sequences were obtained from GenBank. Nucleic acid and protein sequences were aligned using ClustalW (Thompson et al., 1994). The Phylo_win program (Galilei 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'-GGGACAGTTGTTGATACAAAGCAGGGCGCTGGA-3' and the downstream primer 5'-GGGACACATTGTGACCAAAGGTCGTTCACTCAGATAACCCATGACGAGAGGAGTGTG-3'. The PCR amplified cDNA was sequenced to verify that no mutation had been introduced, and subsequently transferred into the pHN65A vector destination (accession no. 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 moiety using thrombin (GE Healthcare-Amersham). Purified recombinant ROMT was incubated in a final volume of 25 μL with 35 μCi [14C]SAM and 200 μM to 1 μM of stilbene substrates in 0.1 Tris, pH 7.5, 20% glycerol (v/v), 5 mM MgCl₂, and 14 μM imidazole. Reaction products were analyzed by TLC on silica gel (Merck) with dichloromethane:acetone (40/3 v/v) as the solvent, using a Molecular Imager FX phosphorimager (Bio-Rad). Enzyme reaction products were identified by comigrating with standards.

Transient Expression in Tobacco

For Agrobacterium-mediated transient expression, ROMT cDNA was trans-

Real-Time Quantitative RT-PCR

Transcript levels were determined by real-time one-step RT-PCR using iCycler IQ thermal cycler (Bio-Rad), and QuantiTect SYBR Green RT-PCR kit (Qiagen). 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). 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 RT, using the RevertAid moloney murine leukemia virus reverse transcriptase (Fermentas), with 0.5 μg of oligo(dT)₁₅ for 1 h at 42°C. PCR amplifications were performed on 5 μL of the 10× diluted cDNA solution using Taq DNA Polymerase from Promega, with 28 cycles of 94°C for 15 s, 55°C for 30 s, and 72°C for 30 s. Primers are described below. All PCR products were separated on 2% agarose gel stained with EB 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).
calculated using the standard-curve method and normalized using grapevine actin gene as internal control. Results presented are means of duplicate data of one representative experiment. The following primers were used for RT-PCR amplifications: 5′-GTGCCAATTGATGAGTGGTGC-3′ and 5′-CCTTCCTAGTTAGAATCTCTAGC-3′ for grapevine actin (accession no. AF369525), 5′-GGGATTTTACGTTAGTATACT-3′ and 5′-CATAGAATAGAAAGC-GCGAGG-3′ for PAL (CF511227), 5′-AAGAAGACGGAACGACAGCAG-3′ and 5′-TGCCCTCTCTCCCTCCCTAATA-3′ for STS (X76902), 5′-TGCCCTCTAG-GCTCCTCTCTA-3′ and 5′-TTGAAAACAGACCTAGCA-3′ for ROMT (FM178870). For P. viticola was amplified using the primers 5′-GTCGACAGCTCAAGCTGC and 5′-CATAATGCTGTCGAACGT GC. To design the primers, actin sequence from the Oomycetes Phytophthora infestans, Pythium splendens, and Achaty biseuisala were aligned together with actin from grapevine. Primer targeted sequences conserved in all three oomycetes, but divergent in grapevine. Absence of amplification in healthy grapevine samples confirmed the specificity of the primers. Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession number FM178870.

ACKNOWLEDGMENTS
We would like to thank Jean-Pierre Dutasta and Jean-Christophe Mulatier (Laboratoire de Chimie, Ecole Normale Superieure de Lyon, France) for help with resveratrol methyl ether synthesis. We are indebted to Prof. Peter Beyer (Laboratoire de Chimie, Ecole Normale Superieure de Lyon, France) for help with ozone treatment, wounding, and fungal infection. Plant Physiol (2000a) Molecular cloning and functional expression of a stress-induced multifunctional O-methyltransferase from Scots pine (Pinus sylvestris L.). Plant Mol Biol Interact 16: 1118–1128

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