Molecular Basis of Ergosterol-Induced Protection of Grape Against *Botrytis cinerea*: Induction of Type I LTP Promoter Activity, WRKY, and Stilbene Synthase Gene Expression

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Type I lipid transfer proteins (LTPs) are basic, 9-kDa cystein-rich proteins believed to be involved in plant defense mechanisms. A 2,100-bp fragment containing the coding region of *Vitis vinifera* lipid transfer protein 1 (*VvLTP1*) and 1,420-bp of its promoter region was isolated by screening a grape genomic library. In silico analysis revealed several putative, defense-related, *cis*-regulatory elements such as W- and MYB-boxes, involved in the binding of WRKY and MYB transcription factors, respectively. The 5'-truncated versions of the *VvLTP1* promoter were generated, cloned in front of the β -glucuronidase (GUS) reporter gene, and introduced in tobacco plants and grapevine cell suspensions using *Agrobacterium* spp. Single MYB- and the W-boxes identified on the 0.250-kbp fragment were sufficient to induce GUS activity in transgenic tobacco plants after transient expression of MYB and WRKY. Ergosterol, a nonspecific fungal elicitor, induced GUS activity in transgenic grapevine cell suspensions transformed with the 1,420- and 750-bp promoter containing a palindromic arrangement of two W-boxes but not the 650- or 250-bp fragment, where only one W-box was present. Moreover, ergosterol triggered *WRKY*, *VvLTP1*, and stilbene synthase gene expression in grape plantlets and enhanced protection against *Botrytis cinerea*. The molecular basis of ergosterol-induced protection is discussed.

Additional keyword: phytoalexin.

Grape (*Vitis vinifera* L.) is the most important fruit crop in the world, with total vineyard acreage of over 19 million acres. This crop is particularly sensitive to various pathogenic fungi, such as *Botrytis cinerea*, the causal agent of gray mold, which can decrease grape berry production and quality. This pathogen is controlled mostly by fungicide treatments, which may cause the appearance of resistant strains and may induce negative environmental impacts (Leroux et al. 1998). The induction or strengthening of natural plant defense mechanisms by elicitors provides an attractive and powerful alternative to current chemical methods (Klarzynski and Fritig 2001). Most of the data about defense responses analyzed in *V. vinifera* upon elicitor treatment fall into two categories: the so-called early events (ionic fluxes, oxidative burst, and phosphorylation events) and

the long-lasting expression of pathogenesis-related (PR) protein and the production of phytoalexins belonging to the stilbene family. BcPG1, an endopolygalacturonase from *B. cinerea*, acts as an elicitor and is able to trigger early defense responses in grapevine suspension cells (Poinssot et al. 2003). Ergosterol, the main sterol of fungi, also was shown to possess elicitor properties in plants (Granado et al. 1995; Kasparovsky et al. 2003, 2004). Ergosterol causes a transient and rapid alkalinization of the growth medium in suspension-cultured tomato (*Lycopersicon esculentum* Mill.) cells (Granado et al. 1995). In tobacco cells (*Nicotiana tabacum* L. cv. Xanthi), ergosterol triggers active oxygen species (AOS) synthesis, changes in ions fluxes, and production of the phytoalexin capsidiol (Kasparovsky et al. 2003). The signal pathways induced after ergosterol recognition depend on internal calcium stores and seem to involve phospholipase A2 (PLA2) and protein kinases (PK) (Kasparovsky et al. 2004). No information currently is available about the defense genes that could be induced by ergosterol. However, induction of class 3 (chitinases), class 2 (β -1,3-glucanases), or class 10 PR-proteins gene expression has been demonstrated during grapevine–pathogen interactions, as well as the production of the phytoalexin resveratrol (Busam et al. 1997; Robert et al. 2001, 2002).

Recently, we have shown that *PR-14* genes encoding non-specific lipid transfer proteins (nsLTPs) are expressed in grapevine cells upon elicitor treatment with either the endopolygalacturonase from *B. cinerea* BcPG1 or with ergosterol (Gomès et al. 2003). nsLTPs first were identified 30 years ago as small, basic, cystein-rich proteins able to transfer lipids between membranes in vitro (Kader 1975). They are encoded by multi-genic families and divided into two classes on the basis of their molecular mass: type I (9 kDa) and type II (7 kDa) nsLTPs (Arondel et al. 2000; Douliez et al. 2001). Their physiological function is still a matter of debate, although they are believed to take part in several biological processes, including somatic embryogenesis, cutin formation, flowering, and cold and salt stress responses (Kader 1996, 1997). Besides these various biological functions, there is a rapidly growing body of evidences linking nsLTPs and plant defense reactions. Transcripts encoding nsLTPs are accumulated in plants challenged by fungi or viruses (Guiderdoni et al. 2002; Molina and Garcia-Olmedo 1993; Sohal et al. 1999), and transgenic tobacco or *Arabidopsis* plants overexpressing a barley *nsLTP* gene were more tolerant to plant pathogens (Molina and Garcia-Olmedo 1997). It has been proposed that this tolerance may be due to

the antibiotic properties of nsLTPs (Garcia-Olmedo et al. 1995). Indeed, various nsLTP-like polypeptides exhibit antifungal or antibacterial activity, at least in vitro (Cammue et al. 1995; Regente and De la Canal 2000; Terras et al. 1992). However, it is unlikely that such a direct antibiotic effect takes place in planta (Gomès et al. 2003); instead, recent evidence points to a role of nsLTPs in plant defense signaling pathways.

An *Arabidopsis* T-DNA-tagged mutant *dir1-1* (defective in induced resistance 1.1) affected on a type II nsLTP gene was unable to develop systemic acquired resistance (SAR) against *Pseudomonas syringae* (Maldonado et al. 2002). A tobacco type I nsLTP bound the defense signaling molecule jasmonic acid and the resulting complex was able to interact with a plasma membrane-located receptor (Buhot et al. 2004). This receptor, previously characterized as the elicitor receptor, is involved in the hypersensitive response (HR) triggered by elicitor treatment in tobacco. Moreover, exogenous application of the LTP–jasmonic acid complex on tobacco plants confers protection against infection by *Phytophthora parasitica* (Buhot et al. 2004). Taken together, these data indicate that induction of nsLTPs gene expression by fungal-derived elicitor molecules could be a viable alternative strategy to enhance tolerance of crops of economic interest, such as grapevine, toward pathogenic microorganisms.

In this context, the present article investigates the regulation of the expression of a grape LTP gene, *Vitis vinifera lipid transfer protein 1* (*VvLTP1*), in response to ergosterol and the involvement of WRKY and R2R3MYB (Stracke et al. 2001) transcription factors in establishment of grape defense responses. We also demonstrate that exogenous application of ergosterol on grapevine plantlets promotes the expression of *VvWRKY1*, *VvLTPs*, and *VST1* (stilbene synthase), and leads to enhanced production of resveratrol and tolerance toward *B. cinerea*.

RESULTS

Isolation and sequence analysis of the *VvLTP1* genomic clone.

Using the full-length *VvLTP1* cDNA (Gomès et al. 2003) as a probe, 20 positive phages were obtained out of 600,000 clones after the third round of screening of the genomic library. After digestion with *SacI*, *XhoI*, and *EcoRI*, the generated fragments were cloned in pBluescript II KS vector (Stratagene), sequenced, and compared with a database using the BLAST program (Altschul et al. 1990). A particular 2,100-bp *EcoRI* fragment contained a 360-bp open reading frame matching the *VvLTP1* cDNA sequence, interrupted by a 110-bp intron located 6 bp upstream from the stop codon (Fig. 1). In addition, this fragment also contained 1,420 bp of promoter region and 210 bp of terminator region. The nucleotide sequence has been deposited in the GenBank database (accession no. DQ136029). Computer analysis of 1,420 bp of the 5' flanking region of *VvLTP1* gene with the PLACE database allowed the identification of several potential *cis*-regulatory elements (Fig. 1). A characteristic TATA box (CTATATAACA) (Messing et al. 1983) and a putative transcription start sites (TCCATCA) (Joshi 1987) were found at positions –106 and –74 from the translation initiation codon, respectively. Several putative regulatory motifs homologous to *cis*-acting elements involved in activating plant defense genes also were identified in the 1,420-bp promoter region of the *VvLTP1* gene. These included two W-boxes (TTGAC, positions –230 and –691, with the box at position –230 being on the complementary strand) (Yu et al. 2001) and one elicitor-responsive element (EIRE) which was W-box-like (position –691, TTGACC) (Rushton et al. 1996). Four regulatory sequences with MYB motifs also were found in the

VvLTP1 promoter: an MYBST1 (GGATA, at –1,388 bp on negative strand (Baranowskij et al. 1994), an MYB1AT (AAACCA, at –1,382 bp on positive strand) (Abe et al. 2003), and two MYBcore (CNGTTG, at –549 and –177 bp on negative strand) (Urao et al. 1993). Finally, one ABA-responsive element (ABRE, ACGTG, at –1,264 bp on negative strand) (Simpson et al. 2003) involved in abiotic stress-related gene activation also was present in the *VvLTP1* promoter region.

To test whether the putative regulatory elements identified by in silico analysis in the *VvLTP1* promoter region were indeed functional, reporter gene assays were carried out. The full-length promoter (1,420 bp) as well as three 5' deletions (750, 650, and 250 bp) were fused with the β -glucuronidase (*GUS*) coding region and named *p1.4VvLTP1-GUS*, *p0.750VvLTP1-GUS*, *p0.650VvLTP1-GUS*, and *p0.250VvLTP1-GUS*, respectively (Fig. 2). These chimeric constructs were introduced in *Agrobacterium* spp. and used to transform grapevine cell suspensions and tobacco plants.

Induction of *VvLTP1* promoter activity in grapevine cell suspension by ergosterol.

Previously, we have shown that *VvLTP1* mRNA strongly accumulates in ergosterol-elicited grapevine cells (Gomès et al. 2003). To analyze the effect of ergosterol on *VvLTP1* promoter activity, different ergosterol concentrations were added directly to grapevine cell suspensions harboring the *p1.4VvLTP1-GUS* construct. Addition of 200 μ M ergosterol doubled the *GUS* activity measured in these cells compared with an untreated control (Fig. 3). Lower ergosterol concentrations had no significant effect on *VvLTP1* promoter activity (data not shown). To determine *cis*-elements that could be involved in the *VvLTP1* promoter induction by ergosterol, the same treatments were performed on grapevine cell suspensions harboring the *p0.750VvLTP1-GUS* and *p0.650VvLTP1-GUS* constructs. The *p0.750VvLTP1-GUS* construct showed the same sensitivity to ergosterol as with the *p1.4VvLTP1-GUS* construct, but no induction was observed with the *p0.6VvLTP1-GUS* construction (Fig. 3). This suggests that one W-box/EIRE present in *p0.750VvLTP1* but absent in *p0.650VvLTP1* (Fig. 2) may be responsible for *VvLTP1* promoter activation by ergosterol. Alternatively, induction of the promoter may require both the W-Box/EIRE at position –691 and the W-box at position –230.

Induction of *VvLTP1* promoter activity by transient expression of MYB and WRKY proteins in tobacco.

Ergosterol induced *VvLTP1* promoter fragments measuring 1.4 and 0.750 kbp of the *VvLTP1* promoter. In this part of the promoter, two W-boxes and one MYB-core motif were present. It is well known that WRKY and MYB factors interact as transcription factors with such DNA motifs (Ülker and Somssich 2004). Many sequences corresponding to these proteins were identified in the *Arabidopsis* genome (at least 70 genes, according to the TAIR database). Therefore, the effect of such elements was checked on the *VvLTP1* promoter. Three grape sequences (*VvMYB2*, *VvMYB4*, and *VvWRKY1*) were available in databases at the beginning of this work and were used to study the interaction of WRKY and MYB proteins with *VvLTP1* promoter in planta. A coexpression system was developed. *VvMYB2*, *VvMYB4*, and *VvWRKY1* cDNAs placed under the control of the 35S RNA promoter of *Cauliflower mosaic virus* were cloned in the pGIBin19 binary vector and introduced in *Agrobacterium tumefaciens*. Transient expression assays were performed by infiltrating young leaves of tobacco plants transformed with the *p0.250VvLTP1-GUS* reporter gene construct with a suspension of *A. tumefaciens* (Fig. 4A). This particular deletion of *VvLTP1* promoter was chosen because it contained

only one W-box and one MYB-box. In each independent experiment, two different clones were used and two leaves per plants were agroinfiltrated. Three different controls were performed in parallel on the same leaf: infiltration with buffer, in-

filtration with untransformed *Agrobacteria*, and the blank (i.e., untreated leaf). The GUS fluorimetric assays were monitored on the samples corresponding to the different leaf areas infiltrated. Expression of *VvMYB4* or *VvWRKY1* transcription fac-

EcoR I		MYBST1 ATAGG (-)	
-1425	GAATTC GTCTACCCACATGTAAGACTTACGATGTTAT TATCCTAAACCA TTTATATGAAC	-1366	
		MYBIAT	
-1365	AGTATTAATCCCCAAATTGATGATAAATATATATATACCGAGACAAAATTAAAGAAAAAA	-1306	
		ABRE GTGCA (-)	
-1305	ACAAGGATTAAGGGTTGCTGGTTGCCCTAGGTTTAA CACGT AATAAGTAGTTGAAAACGA	-1246	
-1245	CCTAGATTTGAAATTTAGACCGGCCACCCACCCTAAATGGTAGTCTATAATCTTGAGCAT	-1186	
-1185	GGCATTGTGGTTGTAAGCTACGGAATTGAATGATTCTCTAATATAAACATCTTCAA	-1126	
-1125	TTAATATATTAATAGCATATAATAATTCCTTGATGAGCGTGTCTCTAATATTCAACAAG	-1066	
-1065	TATATATTAATAAATAAATAAATTAAGGTTGTTTACTTAGGGTTCAAGGTTAAAGTTA	-1006	
-1005	TAAATTATTTTCATCAATCTTTTAAAGAAATCTGTGAATATGATTTTTTTCTAATCTCT	-946	
-945	TTCTATTCATTATTTTTTGAGATGATGGATTTTACCTCAAGTATTGAGATATATTTATA	-886	
-885	TAAGTTTTTAAATAAATAAATTAGTAAGGATTTGATTATTTATATACCCAGTTTATTG	-826	
-825	AAGAGAAATTTTTTTTATAGATTTTACCTTCATTTGAAAGGTTTTTTTACCATTATAT	-766	
-765	TTAATATACTTTTTTGTGAATATTCTTAAATTATTATTAGTCAATTCTTGTAAATTGT	-706	
		ELRE	
-705	TATCATTATGAAGT TTGACC AGGTGAATAATCTAAATATTTCCCTTTTAACTTATCAAC	-646	
		W-Box	
-645	AATTAATGATTATGTTTTTAAATACATTTAAACACAAATTAAAAGCATGGGAAGTTCT	-586	
		GTTGNC MYB-core (-)	
-585	CAAATAAACTTTTATTTTTTAAATATCAT CAACCG TTTTTTAAATTTATTTTAAAAAA	-526	
-525	TTCTTCCAGCAACAAAAGCTCGATGTTTAAATATTAATAATATTACTCTTTTAGTATCGT	-466	
-465	ATTGAATATTTTCGTCCAATTTCACTCAACATGAATAAAATGTGAAGTATGCCACCAAG	-406	
-405	AAATGGAAAACGCTGGAAGTATTAAGGAAAAAAAAAAAAAAAAAGGCCAATGGTACAAAATA	-346	
-345	TATTCATTCCTTTTATAATATTAATTATTTAAATATACAAAGGGGAATTGATATCAAAA	-286	
		W-Box CAGTT (-)	
-285	GTTGGGTGTCGATCCTCCATGATTTATCCGCATCTCTACCGTGAAATGCAT GTCAA ACTA	-226	
		GTTGNC MYB-core (-)	
-225	CACATTGAAAAGTAAGCAAGCGCATTAATCACGGATTAAT CCA ACTGCCAACCAATTC	-166	
-165	CTCCCCATCTCTCATGCATGCCCTTACTGAGTGGTGCCCGAGACCCACCACTTTCTCTAT	-106	
-105	ATAAA ACATCCCTCGTTGAAACCTTT TCCATCAT CACTCTCCAGACCTTCACTTTCTCT	-45	
-44	TCTCTCCTAGCCTTACTAATTTTCTCAGACACTGAGTAGTCATC	ATG GGT AGC TCC	+12
		G A V K L A C V M V I C M V V	
+13	GGA GCT GTG AAG CTA GCT TGT GTG ATG GTG ATA TGC ATG GTG GTG	A A P A A V E A A I T C G Q V	+57
+58	GCG GCA CCG GCG GCG GTG GAA GCA GCC ATA ACA TGT GGT CAG GTG	A S S L S Q C I N Y L Q K G G	+102
+103	GCA TCT TCC TTG AGC CAG TGC ATT AAC TAC TTG CAG AAA GGT GGT	A V P P G C C S G I K S L N S	+147
+148	GCA GTG CCA CCT GGG TGC TGC AGC GGG ATT AAG AGC CTC AAC AGC	A A K T T G D R Q T A C K C L	+192
+193	GCG GCT AAG ACC ACA GGT GAT CGC CAG ACC GCT TGT AAG TGC TTG	K T F S S S I S G I N F G L A	+237
+238	AAA ACC TTT TCT AGT TCC ATC TCT GGC ATC AAT TTC GGT CTT GCA	S G L P G K C G V S V P Y K I	+282
+283	AGT GGG CTT CCG GGC AAG TGT GGT GTC AGC GTT CCT TAC AAG ATC	S P S T D C S K	+327
+328	AGC CCC TCC ACC GAC TGC TCC AAG TAT GCT CTA TTA ATC TCT CAC		+372
+373	TAC TCA TGA TAT ATT TAT ATA GAA TTC AGC TTG ATT AAC CAA TAA		+417
+418	GAT TTC TGC GAC CGA ATT TTT TTT TTT TTG TAA ATG TGT TCA GG		+461
		V T *	
+462	GTG ACT TGA AGC AAA AGG TGG AGG AGT GGG ATG AGC TAT GGT AGC		506
+507	AAC TAG CAT AAG AAT AAA GAA GTG GAG GGT AGA GGC TCC ACT TGG		+551
+552	ACC ACT CAG CCT TTA GTA TTA GTA GTA CTA GTA CTG GTG TCA TAT		+596
+597	TCC TCT CTG TGC TTG TAT GAC ATC TAC TGG AGC GGT TGC ATA CTT		+641
+642	GCA TGT AAT AAA TAT CCC TGC TGG TTG CTG GAC CCT ATC		+680

Fig. 1. Nucleotide sequences of grape *Vitis vinifera* lipid transfer protein 1 (*VvLTP1*) gene and putative cis-acting elements of its 5' upstream region. The A nucleotide of the ATG translation initiation codon is assigned as position 1, and the nucleotide positions upstream of position 1 are presented with negative number. The intron sequence is shown in italic. The deduced amino acid sequence is shown above the nucleotide sequence. The motifs with a significant similarity to previously identified defense-related cis-acting elements are underlined and in bold type. The conserved sequence of the putative transcription start site motif is boxed, with the translational start site shown in bold type. The TATA box is double underlined. An asterisk marks the stop codon.

tors induced a 12- to 14-fold increase in GUS activity conferred by *p0.250VvLTP1*, whereas expression of *VvMYB2* triggered an eightfold increase (Fig. 4B). This induction is in agreement with the presence of one W-box and MYB-box on the 0.250-

Fig. 2. Schematic representation of *Vitis vinifera* lipid transfer protein 1 (*VvLTP1*) promoter regions used for the constructs. Successive deletions of the distal part of the promoter (*p1.4 VvLTP1*, *p0.750 VvLTP1*, *p0.650 VvLTP1*, and *p0.250 VvLTP1*), with restriction sites for restriction enzymes and primers used for chimeric *p VvLTP1/β-glucuronidase (GUS)* fusions are shown. The positions of putative cis-elements are shown with respect to the translation initiation start. Position above or below the promoter line stands for plus or minus strand, respectively.

Fig. 3. β -Glucuronidase (GUS) activity (pmoles MU mg protein⁻¹ min⁻¹) in grapevine 41B cell suspensions carrying *p0.6 Vitis vinifera* lipid transfer protein 1 (*VvLTP1*)/GUS, *p0.7 VvLTP1*/GUS, and *p1.4 VvLTP1*/GUS in response to the addition of ergosterol at 200 μ M (Ergo 200, black bars). For each construct, the control is the same transformed cell suspension treated with ethanol (control, white bars). The compounds were added 4 days after the beginning of subculture and measurements were made 2 days later. Data are the mean $\pm$ standard deviation of three independent experiments. Values differing significantly (Student's *t* test, *P* = 0.05) from the corresponding control are indicated by asterisks. MU: 4-methyl-umbelliferone.

kb-length *VvLTP1* promoter (Fig. 2). This induction was not due to a biotic stress (*Agrobacterium* sp. infiltration) or an abiotic stress (infiltration procedure), because no induction was detected in the different control samples. This in planta analysis of the target promoter and the trans-acting factors revealed a positive interaction between MYB4, MYB2, or WRKY1 proteins and the *VvLTP1* promoter. Infiltration with *Agrobacteria* carrying no effector construct (empty vector) performed on leaves of tobacco plants transformed with *p1.4VvLTP1-GUS*, *p0.750VvLTP1-GUS*, and *p0.650VvLTP1-GUS* constructs triggered the same level of GUS activity as for *p0.250VvLTP1-GUS* induced by infiltration of *Agrobacteria* carrying MYB and WRKY1 effector constructs (data not shown). This induction of GUS activity probably is due to *Agrobacteria* (biotic stress) and did not allow the measurement of MYB or WRKY1 effects on these longer *VvLTP1* promoters.

In order to confirm the involvement of *VvWRKY1* transcription factor in the regulation of *LTP1* gene expression, the capacity of this protein to interact with *LTP1* promoter was tested by electrophoretic mobility shift assays (EMSA). *VvWRKY1* protein was synthesized by in vitro transcription and translation assay and synthetic oligonucleotides probes named LTPW1

Fig. 4. In planta interaction of *Vitis vinifera* (*Vv*)WRKY1 and *VvMYB* transcription factors with the *Vitis vinifera* lipid transfer protein 1 (*VvLTP1*) promoter. **A**, Effector and reporter constructs obtained in pGib19 and pbin19 binary vectors, respectively, and introduced in *Agrobacterium tumefaciens*. **B**, Induction of *p0.250VvLTP1* promoter conferred β -glucuronidase (GUS) activity by *VvMYB* and *VvWRKY1* proteins in tobacco transformants. The effects of *VvMYB2*, *VvMYB4*, or *VvWRKY1* transient expression on the *VvLTP1* promoter were compared with three different controls: untreated areas of the same leaf (control), buffer-infiltrated areas of the same leaf (buffer), and areas of the same leaf infiltrated with the *Agrobacterium* without effector constructs (Agro). Data are the mean $\pm$ standard deviation of three independent experiments. Values differing significantly (Student's *t* test, *P* = 0.05) from the control are indicated by asterisks.

and LTPW2 were designed to contain the first and second W-boxes (–230 and –692 bp), respectively, of LTP1 promoter. For each probe (LTPW1 and LTPW2) incubated with VvWRKY1, EMSA revealed one specific shift band corresponding to a low mobility DNA/protein complex (Fig. 5). This band corresponded to high molecular weight complexes, suggesting formation of W-box-binding multimeres, as previously reported by Yang and associates (1999) and Chen and Chen (2000). Several nonspecific signals (due to reticulocyte proteins) also were detected. The W-box binding specificity was tested by competition experiments. In both cases, addition of unlabeled specific competitors (LTPW1 or LTPW2 in 200-fold molar excess) to the

Fig. 5. Interaction of *Vitis vinifera* (Vv)WRKY 1 protein with W-box, tested by gel retardation experiments. Specific W-box binding activity of VvWRKY1 protein. **A**, Nucleotide sequences (lipid transfer protein [LTP]W1 and LTPW2) and their mutant versions (LTPW1m and LTPW2m) used as probes and specific competitors in electrophoretic mobility shift assays (EMSAs). The W-box elements (TTGAC core) are underlined. The mutated nucleotides are in lowercase. **B**, Binding activity of VvWRKY1 transcription factor to the W-box element was determined by EMSA. Probes (LTPW1 and LTPW2) were incubated with VvWRKY1 protein (W1). Competitors were added in 200-fold molar excess. The second lane represents unspecific DNA/protein complex obtained with protein produced in the TnT system (rabbit reticulocyte lysate [R]). Arrow indicates DNA-protein complex.

binding reaction reduced the retarded signal, whereas addition of an unlabeled W-box-mutated LTPW1m and LTPW2m probe (changing TTGAC to TTGAA) to the reaction volume had little effect. The presence of this low-mobility complex suggests that VvWRKY1 transcription factor may form an oligomeric complex which is able to bind specifically the two first W-boxes of the LTP1 promoter.

Induction of VvWRKY1 and VvLTP1 gene expression by ergosterol.

Taken together, the results of agroinfiltration experiments and induction of VvLTP1 promoter activity by ergosterol in grapevine cell suspensions suggest an involvement of VvWRKY1 protein, via its binding to W-box, in VvLTP1 gene upregulation by ergosterol. To test this hypothesis, the expression of VvWRKY1 and VvLTP1 in grapevine plantlets treated with ergosterol was analyzed by RNA blot. VvWRKY1 mRNA level started to increase 2 h after ergosterol treatment, and peaked at 5 h (Fig. 6, upper panel). VvLTP1 transcripts increased at 24 h and kept accumulating until 48 h. These results were confirmed by phosphor-imager quantification of the RNA blot hybridization signals (Fig. 6, lower panel). The hybridization signal was normalized using 28S rRNA signal as a standard. Upon ergosterol treatment, a 23-fold increase of VvWRKY1 gene expression was observed after 5 h, whereas a 4.5-fold increase of VvLTP1 gene expression was observed after 24 h.

Grapevine plantlets pretreated with ergosterol are protected against *B. cinerea* in vitro.

Previous results have shown the ability of ergosterol to elicit defense responses, including AOS production, phytoalexin synthesis (Granado et al. 1995; Kasparovsky et al. 2003, 2004), and induction of PR-14 in 41B grapevine cell suspension (Gomès et al. 2003). To test the ability of this elicitor

Fig. 6. Time-course analysis of *Vitis vinifera* lipid transfer protein 1 (VvLTP1) and VvWRKY1 mRNA accumulation in ergosterol-treated tissue of grapevine (cv. Ugni blanc) plantlets. Leaves were treated with 200 μ M ergosterol and collected 0, 2, 5, 10, 24, or 48 h after treatment. For each time point, three independent plants were pooled for RNA extraction. Upper panel: hybridization signals obtained with 41B-LTP1, VvWRKY1 and rRNA probes. Lower panel: quantification of the hybridization signals with the Phosphor-Imager; ○ = VvLTP1 hybridization signal and △ = VvWRKY1 hybridization signal.

molecule to protect grapevine plantlets against *B. cinerea*, an in vitro protection assay was performed. All leaves of 6-week-old grapevine plantlets were treated with an ergosterol solution (200 μ M ergosterol, 0.2% ethanol, and 0.02% Triton X100) or the solvent (0.2% ethanol and 0.02% Triton X100). The same experiments were performed using a benzothiadiazole (BTH) solution (600 μ M BTH, 0.2% ethanol, and Triton 0.02% X100) as a positive control. BTH, a well-known chemical inducer of SAR, was reported to protect various plant species against bacterial and fungal pathogens (Görlach et al. 1996; Sparla et al. 2004). Fifteen plants were used for each treatment. At 24 h after treatment, elicited and control plantlets were inoculated on the third leaf from the top with 250 conidia of *B. cinerea*. At 15 to 20 days post inoculation (dpi), the first symptom of gray mold (necrosis formation) appeared on the inoculated leaf of some untreated, solvent-treated, and BTH-treated plantlets. No symptoms were noticed on any ergosterol-treated plantlets (data not shown).

At 20 to 40 dpi, 95% of untreated, 92% of solvent-treated, and 45% of BTH-treated plantlets showed necrosis, detachment of the infected leaves, mycelium, and spore formation. Only 25% of the ergosterol-treated plantlets also showed some of these latter symptoms (Fig. 7), meaning that 75% of these plants were protected against *B. cinerea*.

These data indicate that ergosterol application led to the induction of protection against *B. cinerea*.

Ergosterol induces *VST1* gene expression and accumulation of resveratrol.

The role of resveratrol, the main grapevine phytoalexin, in resistance to *B. cinerea* is well documented (Coutos-Thevenot et al. 2001; Robert et al. 2001). To investigate whether the protection induced by ergosterol was associated with resveratrol production, *VST1* gene expression and resveratrol content were analyzed in grapevine plantlets treated with ergosterol. Analyses were made on five independent plants treated with ergosterol or with a 0.2% ethanol and 0.02% Triton X100 solution as a control (the solvent in which ergosterol was solubilized). Ergosterol treatment strongly induced *VST1* gene expression, with a maximum at 10 h post treatment (Fig. 8A), as confirmed by phosphor-imager quantification (Fig 8B). Expression decreased after 10 h and returned to basal level at 24 h. Resveratrol quan-

Fig. 7. Percentage of protection against *Botrytis cinerea* obtained on grapevine (cv. Ugni blanc) plantlets untreated or pretreated with benzothiadiazole (BTH), ergosterol, or solvent (control, EtOH) as a control. For each treatment, 15 independent plants were tested.

tification showed that control plants treated with 0.2% ethanol and 0.02% Triton X100 exhibited a low phytoalexin content (16.5 ± 4.5 ng g⁻¹ fresh weight). This low-level production of stilbene molecules is most certainly due to the solvent, because no resveratrol was detected in untreated grapevine plants (data not shown). Resveratrol accumulated to a high level in ergosterol-treated plants (227 ± 120 ng g⁻¹ fresh weight) (Fig 8C). Standard deviation was relatively high in resveratrol-treated plants, probably due to a large heterogeneity in individual plant response to ergosterol. Nevertheless, the results obtained on five independent ergosterol-treated plants were significant compared with the solvent-treated control plants according to Student's test ($P = 0.05$). Based on these results, it is quite clear that ergosterol induces an accumulation of *VST1* transcripts and triggers the production of resveratrol in treated leaves.

DISCUSSION

To further investigate the regulation of *VvLTP1* gene expression, the *VvLTP1* gene associated with its 5' upstream region was isolated by screening a grapevine genomic library. The DNA structure of the *VvLTP1* gene with a 110-bp intron interrupting the coding sequence two codons upstream of the stop codon is similar to those of other plant *LTP* genes (Kader

Fig. 8. Resveratrol quantification by high-performance liquid chromatography (HPLC) and *VST1* expression analysis in plants treated with ergosterol. Five plants were treated with 200 μ M ergosterol (Ergosterol) or 0.2% ethanol–0.02% Triton X100 solution (EtOH) during 20 h. **A**, RNA blot analysis was performed at different times of ergosterol induction and the membrane was hybridized with *VST1* or rRNA probe. **B**, After hybridization, the RNA blot was quantified using a Phosphor-Imager. **C**, HPLC analysis of resveratrol production was performed after methanolic extraction and the values in the graph represent the mean $\pm$ standard deviation of five independent plants.

1996). In addition to the typical TATA and CAAT boxes, the isolated *VvLTP1* promoter region (*p1.4 VvLTP1*) contains several *cis*-acting elements, including ABRE, MYB, and W-box elements (Figs. 1 and 2) that previously were shown to be involved in the activation of gene expression by ABA, abiotic stress, pathogen, salicylic acid (SA), or elicitor (Abe et al. 2003; Kim et al. 1997; Rushton et al. 1996; Simpson et al. 2003; Yu et al. 2001).

The *VvLTP1* promoter fragments corresponding to 1,420 and 750 bp in length, but not the one corresponding to 650 bp in length, were activated by 200 μ M ergosterol in stably transformed grapevine cell suspension (Fig. 3). These results are consistent with previous data demonstrating that nsLTP mRNAs accumulate in ergosterol-treated 41B grapevine cells (Gomès et al. 2003). This strongly suggests that the positive *cis*-acting elements inducing the *VvLTP1* expression in grapevine cells in response to ergosterol treatment are located between -1,420 and -650 bp. Interestingly, the *p0.750VvLTP1* contains one additional W-Box if compared with the *p0.650VvLTP1* (Fig. 2). Therefore, this W-box is likely to be involved in induction of *VvLTP1* promoter activity by ergosterol. Previous results demonstrated that fungal elicitor-induced transcription of the parsley *WRKY1* and *PR1* genes is mediated almost exclusively by W-box elements, which are binding sites for WRKY-type proteins (Rushton et al. 1996). Moreover, *WRKY1* itself is expressed rapidly in parsley leaf tissue around pathogens infection sites, and this expression is mediated by a palindromic arrangement of W-boxes in the *WRKY1* promoter region (Eulgem et al. 1999). Such a palindromic arrangement is found in 1.4- and 0.750-kb-length *VvLTP1* promoter regions and could explain the induction of *VvLTP1* by ergosterol. Accordingly, the *p0.650VvLTP1* promoter, which contains only one W-box, is unable to respond to such a treatment.

In planta co-expression of *VvWRKY1* and *VvLTP1* shows that *VvWRKY1* induces GUS activity driven by the *VvLTP1* promoter through its binding to a W-box (Fig. 4). Taken together, these results strongly suggest that *VvWRKY1* is involved in upregulation of *VvLTP1* expression by ergosterol via its binding to a W-box. Moreover, *VvWRKY1* and *VvLTP1* expression kinetics in ergosterol-treated plants are in agreement with this hypothesis: the maximum level of *VvWRKY1* mRNA (at 5 h) precedes the one of *VvLTP1* (at 24 h) (Fig. 6). These results are consistent with previously published results. In a general survey of *WRKY* gene expression in *Arabidopsis thaliana*, Dong and associates (2003) found that 43 of the 72 *WRKY* genes were upregulated after *Pseudomonas* sp. infection or SA treatment. Most of the transcripts started to accumulate as soon as 2 h post treatment, and reached a maximum between 4 and 8 h. This induction precedes the induction of *PR* genes, such as *PR1*, known to be regulated by WRKY transcription factors (Eulgem et al. 1999).

The second part of this study was aimed at testing the ability of ergosterol to stimulate grapevine defense mechanisms. Protection assays showed that an exogenous application of ergosterol protected 75% of the treated plantlets against *B. cinerea* (Fig. 7).

It was tempting to associate this protective effect with *VvLTP1* upregulation. Based on recently published results on the involvement of nsLTP in a defense signaling pathway (Buhot et al. 2004; Maldonado et al. 2002), it seems unlikely that the protection conferred by ergosterol to grapevine plantlets against *B. cinerea* is directly due to *VvLTP1* expression. Based on all these results, it is possible to conclude that ergosterol induces events occurring in the following sequence: i) WRKY trans-acting elements mobilization, ii) stilbene synthase gene expression and resveratrol production, and iii)

VvLTP1 accumulation in plants, the final output being protection against *B. cinerea*. Moreover, it is known that LTP forms a complex with jasmonic acid (JA) and that the LTP-JA complex can bind to the elicitor receptor. Treatment of tobacco plants with the LTP-JA complex induces protection against *Phytophthora parasitica* (Buhot et al. 2004). Assuming this sequence of events, a possible involvement of LTPs in defense mechanisms may be proposed. It is quite clear that ergosterol is perceived by plant cells as a nonspecific elicitor inducing a classical defense transduction pathway (Kaparovsky et al. 2003, 2004) leading to the production of phytoalexins and PR-protein induction (PR14, for example). It is likely that *VvLTP1* is upregulated after *WRKY* factor gene expression, and we demonstrated here that *WRKY* and MYB proteins are involved in *VvLTP1* promoter regulation via W-box and MYB. Ergosterol could bind to a plasma membrane receptor and trigger a classical signal transduction pathway, as demonstrated for many nonspecific elicitors. This signaling cascade could induce expression of WRKY trans-acting proteins and local accumulation of stilbenes. In a second step, *VvLTP1* gene expression is induced and the corresponding protein is secreted in the extracellular compartment (Gomes et al. 2003). At the same time, ergosterol could trigger lipoxygenase expression and JA production from membrane fatty acids, as demonstrated for other elicitors (Engelberth et al. 2001; Xiangyang et al. 2003). A long-distance transport of LTP-JA complexes in the conducting bundles could be necessary for establishment of a systemic protection. This last step is far from being demonstrated and is the major interrogation of this pathway, even though the presence of LTP in phloem and xylem saps already has been demonstrated, suggesting the mobility capacities of these proteins (Maldonado et al. 2002; Rep et al. 2003). In the future, the investigation of such a long-distance transport of LTP-JA complexes will be a major goal to achieve.

MATERIALS AND METHODS

Genomic library screening.

A grapevine (cv. Ugni Blanc) genomic library (Fillion et al. 1999) was used to isolate a *VvLTP1* genomic clone. Approximately 600,000 plaques were screened using the full-length *VvLTP1* cDNA probe (GenBank AF465408) (Gomès et al. 2003). Labeling of the probe was performed with 32 [P]-dCTP using the Ready-to-Go DNA labeling kit (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.). Positive clones were identified after three rounds of screening and characterized by restriction analysis and sequencing.

Promoter-GUS fusions construction.

Various lengths of *VvLTP1* promoter were cloned in front of the *uidA* gene coding region encoding GUS in the binary vector pBin 19 (Frisch et al. 1995). A *Bam*HI site was introduced by polymerase chain reaction (PCR) at the 3' end of the promoter region immediately upstream of the *VvLTP1* translation initiation site. Two deletions of *Vv-LTP1* 5' flanking region were obtained as restriction fragments of 250 bp (*Eco*RV-*Bam*HI) and 1,400 bp (*Eco*RI-*Bam*HI) from the entire promoter. The proximal promoter regions of 650 and 750 bp (*Ssp*I-*Bam*HI) were amplified by PCR. The PCR products were subcloned into the pGEM-T easy vector (Promega Corp., Madison, WI, U.S.A.) and confirmed by sequencing. The *VvLTP1* promoters were transcriptionally fused in front of GUS reporter gene. The 35S promoter/GUS fusion was used as a constitutive control, and the promoterless-GUS construct was used as a negative control. Cloning in *Escherichia coli* was performed in strain DH5 α . All promoter-GUS constructs were confirmed by sequencing and introduced by electropora-

tion in *Agrobacterium tumefaciens* EHA 105 for tobacco and grapevine cell transformations.

Tobacco transformation.

Tobacco plants (*Nicotiana tabacum* cv. Samsun NN) were transformed by a leaf disk method modified from that of Horsch and associates (1985). Murashige and Skoog medium was used with one-half NH_4NO_3 concentration and supplemented with kanamycin at $150 \mu\text{g ml}^{-1}$ and cefotaxime at $350 \mu\text{g ml}^{-1}$ (Duchefa, Haarlem, The Netherlands). Calli formation and shoot regeneration were allowed to proceed in the presence of 6-benzylaminopurine (2 mg liter^{-1}), 1-naphthalene acetic acid ($0.05 \text{ mg liter}^{-1}$), and sucrose (30 g liter^{-1}). Rooting was obtained on hormone-free Murashige and Skoog medium at a sucrose concentration of 15 g liter^{-1} . For each construct, from 12 to 20 independent primary transformants were produced. Transformed plants were grown in vitro under a regime of 16 h of light and 8 h of darkness at 22°C .

Agroinfiltration of tobacco plants.

Agrobacteria infiltration was conducted as described by Yang and associates (2000). *A. tumefaciens* EHA105 containing the binary vector pGiBin19 with *VvMYB1* (accession number AY555190), *VvMYB2* (accession number AY899404), or *VvWRKY1* (accession number AY585679) cDNAs under the control of 35S promoter were grown at 28°C overnight in YEB liquid medium (sucrose at 5 g liter^{-1} , yeast extract at 1 g liter^{-1} , bactopectone at 10 g liter^{-1} , and Gibco beef extract at 5 g liter^{-1} , pH 7.4) supplemented with rifampicin ($100 \mu\text{g ml}^{-1}$) and kanamycin ($50 \mu\text{g ml}^{-1}$). Agrobacteria then were inoculated in 20 ml of induction medium containing AB salts ($18.6 \text{ mM NH}_4\text{Cl}$, 1.2 mM MgSO_4 , 1.9 mM KCl , 0.06 mM CaCl_2 , and 0.008 mM FeSO_4), 2 mM phosphate, 1% glucose, 20 mM Mes (pH 5.5), $100 \mu\text{M}$ acetosyringone, and rifampicin and kanamycin at the same concentrations given above. After overnight culture at 28°C , the bacteria were collected by centrifugation (15 min at $3,000 \times g$) and washed once in a solution containing 10 mM Mes, pH 5.5, 10 mM MgSO_4 , and $100 \mu\text{M}$ acetosyringone. Cells then were resuspended in the same solution, and the absorbance at 600 nm of the suspension was adjusted to 0.8 before infiltration. Young, nearly expanded leaves of 6-week-old tobacco plants were infiltrated. The infiltrated areas were collected after 48 h and frozen in liquid nitrogen. The GUS fluorimetric assays were performed according to the method of Jefferson and associates (1987).

Grapevine transformation, culture, and elicitation conditions.

Grapevine transformations were made in 41B rootstock (*V. vinifera* cv. Chasselas $\times$ *V. berlandieri*). An embryogenic cell suspension culture was initiated as described previously (Coutos-Thévenot et al. 1992a,b). This cell suspension was subcultured each week in 25 ml of glycerol-maltose culture medium (Coutos-Thévenot et al. 1992b) supplemented with synthetic auxin (naphthoxy acetic acid at 1 mg liter^{-1}) in the dark. Embryogenic cells were transformed using an *Agrobacterium* cocultivation method (Mauro et al. 1995) and, after selection, the transgenic cells were subcultured in the same condition in a medium supplemented with paromomycin at $2 \mu\text{g ml}^{-1}$ and cefotaxime at $200 \mu\text{g ml}^{-1}$ (Duchefa).

Transgenic cell suspension cultures were treated 4 days after subculture by addition of ergosterol (dissolved in ethanol) directly to the medium. Cells were collected by filtration 48 h after treatment, frozen in liquid nitrogen, and stored at -80°C . The effects of the treatment were measured using fluorimetric GUS assay made on the same suspension cells treated with ergosterol or ethanol (control), or untreated.

Fluorimetric GUS assay.

GUS fluorimetric assays were carried out on 41B transgenic suspension cells after various treatments and on different infiltrated leaves of 6-week-old transformed tobacco plantlets 48 h after treatments according to the procedure of Jefferson and associates (1987). GUS enzyme activity was determined by measuring the kinetics of appearance of MU, produced by cleavage of methylumbelliferyl- β -D-glucuronide. Fluorescence was read on a FluoroCount Microplate Fluorometer (Packard Instruments, Meriden, CT, U.S.A.). GUS enzyme activity was expressed as picomoles of MU produced per minute per milligram of protein. Protein content was determined according to Bearden (1978), using bovine serum albumin as a standard. All GUS measurements presented were confirmed in a total of 12 measurements coming from three independent experiments.

Grapevine plantlets culture and elicitation treatment.

Grapevine plantlets (cv. Ugni blanc) were multiplied by internode microcuttings in Murashige and Skoog culture medium, supplemented with agar at 7.5 g liter^{-1} , pH 5.8, in MAGENTA boxes (Sigma, St. Louis). The microcuttings were maintained at 24°C during the day and 18°C during the night, with 16 h light at $250 \mu\text{E m}^{-2}$. Ergosterol was dissolved in 0.2% ethanol and 0.02% Triton X100 and applied on the leaves of the 6-week-old grapevine plantlets.

RNA extraction and Northern blot analysis.

Ergosterol-elicited plantlets were excised and immediately frozen in liquid nitrogen 0, 2, 5, 10, 24, and 48 h after the treatment. For each time point, three independent plants were pooled and frozen in liquid nitrogen, and total RNAs were extracted from 1 g of plantlet tissues according to Chang and associates (1993), with modifications. Leaves were ground in liquid nitrogen to a fine powder, homogenized in extraction buffer (100 mM Tris-HCl, pH 8.0, 2% cetyltrimethylammonium bromide, 2% polyvinyl-polypyrrolidone, 25 mM EDTA, 2 M NaCl, and spermidin at 0.5 g liter^{-1}), and incubated for 5 min at 65°C . The resulting suspension was extracted twice by one volume of chloroform/isoamyl alcohol (24:1, vol/vol). The aqueous phase was collected and RNAs then were precipitated by adding LiCl (2 M final). After centrifugation, the pellet was dissolved in 0.5% sodium dodecyl sulfate (SDS) and extracted once by chloroform/isoamyl alcohol. The aqueous phase was collected and RNAs were precipitated by addition of 2.5 volumes of ethanol. The pellet was washed once with 70% ethanol, air dried, and dissolved in water. RNA concentration was quantified by measuring absorbance at 260 nm .

Total RNA ($20 \mu\text{g}$) was mixed with a loading buffer (32.7% formamide and 1.21 M formaldehyde) and loaded onto a 1.2% agarose gel containing MEN buffer (20 mM MOPS, pH 7.0, 5 mM sodium acetate, and 1 mM EDTA) and supplemented with 2.25 M formaldehyde. The gels were run for 5 h at 50 V in MEN buffer, and capillary blotted onto a Hybond N nylon membrane (Amersham Pharmacia Biotech) with $10\times$ SSC buffer ($1\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate). RNAs were cross-linked to the membrane by heating for 2 h at 80°C . At this stage, the membrane was stained with methylene blue to check for equal RNA loading on the gel. Hybridization was performed overnight at 65°C in a buffer containing 0.25 M sodium phosphate (pH 7.2), 1% bovine serum albumin, 7% SDS, and 1 mM EDTA. Radiolabeled DNA probes were synthesized by random priming, using the Ready-to-Go DNA labeling beads kit and $[^{32}\text{P}]\text{-dCTP}$ (Amersham Pharmacia Biotech). After hybridization, the membranes were washed at 65°C twice in $2\times$ SSC and 0.1% SDS, twice in $0.5\times$ SSC and 0.1% SDS, and once in $0.2\times$ SSC and 0.1% SDS. Membranes finally were exposed 24 h to phosphoscreens for quantification.

with a Storm Phosphor Imager (Molecular Dynamics, Sunnyvale, CA, U.S.A.). The 28S ribosomal RNA from *Arabidopsis thaliana* was used as a probe to normalize hybridization signals between the different lanes.

In vitro VvWRKY1 protein synthesis and electrophoretic mobility shift assay.

The VvWRKY1 protein was produced by in vitro transcription and translation method with the TnT T7 quick system for PCR DNA system (Promega, Charbonnières, France) according to the manufacturer's instructions. The coding sequence first was amplified with Turbo-Pfu (Stratagene, La Jolla, CA, U.S.A.) using the primer pairs 5'-AGATCCTAATACGACTCACTATAGGGAGCCACCAUGGAAGGCCACCAAATATT-3' and 5'-(T)₃₂GGTGAATGGAAACATTCAT-3'. The resulting PCR product was used as template. Protein detection was performed using the Transcend nonradioactive translation detection system (Promega). Double-strand probes were labeled with [γ -³²P] ATP using a 5' end labeling kit (Amersham Pharmacia Biotech) and purified on a 6% nondenaturing polyacrylamide gel. The resultant 80,000-cpm labeled DNA probe was incubated with 6 μ l of protein reaction mixture, 2 μ g of poly(dI-dC)-poly(dI-dC), and the binding buffer (25 mM Hepes, 30 mM KCl, 0.1 mM EDTA, 10% glycerol) in a 20- μ l reaction volume. In competition experiments, specific competitor was added in 200-fold molar excess prior to the addition of the protein. The mixture was incubated for 30 min at room temperature and analyzed on a nondenaturing 6% polyacrylamide gel in 0.5 $\times$ Tris-borate-EDTA buffer at 160 V. The gel then was dried and autoradiographed using FX Phosphor-Imager (BioRad, Veenendaal, The Netherlands).

Protection assays.

The *B. cinerea* strain 916T was obtained from Dr. B. Dubos (INRA Bordeaux, France) and subcultured each month on malt-agar culture medium (malt extract at 15 g liter⁻¹ and bacto agar at 20 g liter⁻¹) in the dark at 21°C. Conidia were obtained by fungus inoculation on V8 (commercial Campbell's vegetable juice) culture medium diluted to half strength and supplemented with bacto-Agar at 20 g liter⁻¹ under light at 21°C. Six-week-old-grapevine plantlets were treated with an ergosterol solution as mentioned above (200 μ M ergosterol, 0.2% ethanol, and 0.02% Triton X100) and inoculated after 24 h with *B. cinerea* by applying 10- μ l droplets of the conidia suspension (25 conidia/ μ l suspended in sterile distilled water) using a micropipette on the surface of the third leaf from the top. Quantification of disease development after inoculation with *B. cinerea* was measured as extent of necrosis, leaf fall, mycelium, and spore formation.

Resveratrol extraction and quantification.

Frozen leaves were ground with a mortar and pestle in liquid nitrogen and resveratrol was extracted with 0.5 ml of methanol per 100 mg of fresh weight. The methanolic suspension was centrifuged for 10 min at 13,000 $\times$ g. To remove chlorophylls, the supernatant was passed through a Sep-Pack C-18 cartridge (Waters, Milford, MA, U.S.A.) equilibrated with methanol/water 90:10 (vol/vol). The filtrate (180 μ l) was injected with or without purified resveratrol addition as an external standard. Analysis was performed on a series 200 HPLC system (Perkin-Elmer, Branchburg, NJ, U.S.A.) equipped with an automatic injector. Samples (180 μ l of each) were loaded onto a Hypersil ODS C-18 reverse phase column (Phenomenex, Torrance, CA, U.S.A.) equilibrated with an acetonitrile/water/TFA (1:98.9:0.1, vol/vol/vol) mobile phase (solvent A) at a flow rate of 0.8 ml min⁻¹. The bound molecules were eluted with a linear gradient of solvent B (acetonitrile/H₂O:TFA,

80:19.9:0.1, vol/vol/vol). The elution gradient was as follows: 0 to 30 min, 100% of B. Elution was monitored by absorbance at 305 nm, the optimal absorption of resveratrol. Quantification was obtained after injection of increasing quantities of 3,4',5-trihydroxy-trans-stilbene (99% purity), the natural resveratrol (Sigma, Lyon, France), to establish the calibration curve as a function of integrated peak area. Results were expressed as nanograms of resveratrol per gram of fresh weight.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

PLACE, a database of plant *cis*-acting regulatory DNA elements:
www.dna.affrc.go.jp/PLACE

TAIR, the *Arabidopsis* information resource: www.arabidopsis.org