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Pedro Guillén, Monique Guis, Gracia Martinez-Reina, Ségolène Colrat, Sylvie Dalmayrac, et al.. A novel NADPH-dependent aldehyde reductase gene from *Vigna radiata* confers resistance to the grapevine fungal toxin eutypine.. *Plant Journal*, 1998, 1 (3), pp.335-343. 10.1046/j.1365-313x.1998.00303.x. hal-03485305

HAL Id: hal-03485305

<https://hal.science/hal-03485305>

Submitted on 17 Dec 2021

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A novel NADPH-dependent aldehyde reductase gene from *Vigna radiata* confers resistance to the grapevine fungal toxin eutypine

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Summary

Eutypine, 4-hydroxy-3-(3-methyl-3-butene-1-ynyl) benzyl aldehyde, is a toxin produced by *Eutypa lata*, the causal agent of eutypa dieback of grapevines. It has previously been demonstrated that tolerance of some cultivars to this disease was correlated with their capacity to convert eutypine to the corresponding alcohol, eutypinol, which lacks phytotoxicity. We have thus purified to homogeneity a protein from *Vigna radiata* that exhibited eutypine-reducing activity and have isolated the corresponding cDNA. This encodes an NADPH-dependent reductase of 36 kDa that we have named *Vigna radiata* eutypine-reducing enzyme (VR-ERE), based on the capacity of a recombinant form of the protein to reduce eutypine into eutypinol. The strongest homologies (86.8%) of VR-ERE at the amino acid level were found with CPRD14, a drought-inducible gene of unknown function, isolated from *Vigna unguiculata* and with an aromatic alcohol dehydrogenase (71.7%) from *Eucalyptus gunnii*. Biochemical characterization of VR-ERE revealed that a variety of compounds containing an aldehyde group can act as substrates. However, the highest affinity was observed with 3-substituted benzaldehydes. Expression of a VR-ERE transgene in *Vitis vinifera* cells cultured *in vitro* conferred resistance to the toxin. This discovery opens up new biotechnological approaches for the generation of grapevines resistant to eutypa dieback.

Introduction

The ascomycete fungus *Eutypa lata* is the causal agent of eutypa dieback of grapevines (Munkvold *et al.*, 1994). The pathogen infects *Vitis vinifera* through pruning scars, colonizes the xylem tissue, and finally propagates into the cambium and phloem of the trunk and branches (Duthie *et al.*, 1991). It has been suggested (Deswarte *et al.*, 1996a) that during the growth period of grapevine, eutypine [4-hydroxy-3-(3-methyl-3-butene-1-ynyl) benzyl aldehyde], a toxin synthesized by the mycelium (Mauro *et al.*, 1988; Renaud *et al.*, 1989; Tey-Rulh *et al.*, 1991), is transported from the trunk through the sap to the herbaceous parts of the vine where it induces a number of symptoms including dwarfed and withered shoots, marginal necrosis of the leaves, dry and shrivelled inflorescences and, finally, death of the whole branch. So far, no active chemicals are available against *Eutypa lata*. Only expensive and time consuming methods including cultural practices or localized chemical applications have been used to limit pathogen development. However, these techniques are inefficient and therefore eutypa dieback is presently one of the most serious fungal diseases facing viticultural industries (Moller and Kasimatis, 1981; Munkvold *et al.*, 1994).

Eutypine plays an important role in the expression of the disease (Fallot *et al.*, 1997). *In vitro* studies have shown that the toxin is rapidly taken up by grape cells through passive diffusion and tends to accumulate in the cytoplasm due to an ion trapping mechanism (Deswarte *et al.*, 1996a). Furthermore, eutypine uncouples mitochondrial oxidative phosphorylation by increasing proton leakage through a cyclic protonophore mechanism (Deswarte *et al.*, 1996b). The level of sensitivity to eutypa dieback depends on the genotype of the host (Péros and Berger, 1994). Following this observation, we have established that the tolerance of some grape genotypes is related to their capacity to detoxify eutypine into its corresponding alcohol, eutypinol [4-hydroxy-3-(3-methyl-3-butene-1-ynyl) benzylic alcohol], which lacks phytotoxicity. This reaction has been shown to be NADPH-dependent (Fallot *et al.*, 1997). We found that metabolism of eutypine was not restricted to grapevine varieties but that several plant species are also capable of converting eutypine into eutypinol, among which *Vigna radiata* exhibited the highest activity. We have undertaken the purification to homogeneity of an eutypine-reducing enzyme from *Vigna radiata* (VR-ERE). We report here the isolation of the corresponding cDNA, its functional expres-

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Table 1. Isolation of the full-length cDNA from peptide sequences. Degenerate oligonucleotides MBn1 and MBn5 used for PCR correspond to the underlined peptidic sequences MBp1 and MBp5

Internal peptide sequences		Oligonucleotide sequences	
MBp1	SLGID <u>Y</u> IPLE <u>V</u> SL	MBn1	GAYTAYATHCCNYTNGARGT
MBp2	TLAEDA <u>A</u> AWK		
MBp3	GTLN <u>V</u> LK		
MBp4	NASLGWVDVK		
MBp5	ANLLEEGSFDSAVEGVH <u>A</u> V <u>F</u> H <u>T</u> A <u>X</u>	MBn5	GTNCAYGCNGTNTTYCAYAC

Y = C,T; R = A,G; H = A,T,C; N = A,T,C,G.

sion in *Escherichia coli*, and the evaluation of eutypine resistance of transgenic grape cells over-expressing this gene. The outcome of this discovery is discussed in terms of disease prevention through genetic engineering approaches.

Results and discussion

Isolation and characterization of VR-ERE cDNA clone from *Vigna radiata*

We have previously shown that grapevine tissues have the capacity to convert eutypine to eutypinol through an NADPH-dependent enzyme activity (Fallot *et al.*, 1997). However, even in tolerant genotypes, the eutypine-reducing activity of grapevine is low. Since eutypine is a non-specific protonophore that causes cell death in a wide variety of cells, including mammalian (Deswarte *et al.*, 1996b), we speculated that tissues other than grapevine could exhibit eutypine-reducing activity. A screening of several plant tissues led to the discovery that mung bean (*Vigna radiata*), a non-host plant of *Eutypa lata*, exhibited an eightfold higher eutypine-reducing activity than grapes and was therefore a suitable material for purifying to homogeneity an NADPH eutypine-reducing enzyme. The purification procedure described in detail in the Experimental procedures combined $(\text{NH}_4)_2\text{SO}_4$ fractionation, hydrophobic chromatography on phenyl-sepharose, ion exchange chromatography on hydroxyapatite, exclusion chromatography on Superose 12 HR, anionic exchange chromatography on Mono-Q, and finally separation on SDS-PAGE. This procedure allowed the isolation of a protein of 36 kDa that was subjected to endo-proteinase Lysine C digestion and partial amino acid sequencing. The five internal peptide sequences are reported in Table 1. Degenerate oligonucleotides designed from two of these peptides (peptides MBp1 and MBp5, Table 1) were used to amplified by PCR a partial VR-ERE cDNA clone of 0.7 kb. The full-length clone (1254 bp) was finally isolated by a combination of inverse PCR and PCR. It consisted of an open reading frame of 975 nucleotides starting at ATG and ending with GTT, 995 bp upstream of the poly(A)⁺ tail. The

deduced amino acid sequence (Figure 1) corresponds to a 325 amino-acid polypeptide with an expected molecular mass of 35.9 kDa and an isoelectric point of 6.34. All internal peptide sequences obtained from the purified polypeptide were found in the deduced peptidic sequence (not shown).

A search of protein sequence databases (Figure 1) showed that the VR-ERE cDNA encodes a protein that shows highest homologies with a drought-inducible gene, CPRD14 (86.8% identity at the cDNA-deduced amino acid level), of unknown function isolated from *Vigna unguiculata* (luchi *et al.*, 1996) and the CAD1 cDNA of *Eucalyptus gunnii* encoding an aromatic alcohol dehydrogenase, also considered as a cinnamyl alcohol dehydrogenase (71.7% identity, Goffner *et al.*, 1998). The VR-ERE protein exhibited lower homology (47.1% identity) with a cinnamoyl-CoA reductase (CCR) from *Eucalyptus gunnii* (Lacombe *et al.*, 1997) and several proteins encoding for dihydroflavonol reductase (DFR, the greatest being 44.6% identity with DFR of *Dianthus caryophyllatus*). However, the phylogenetic tree (Figure 2) clearly established a separation between the VR-ERE protein and the DFR and CCR family. It showed that CPRD14, CAD1 and VR-ERE were closely related and could be classified within an aldehyde reductase superfamily. This new branch of enzymes emerging from the phylogenetic tree can be considered as belonging to the superfamily of NADPH-dependent reductases, established by Baker and Blasco (1992), that gather plant DFR and mammalian 3- β -hydroxysteroid dehydrogenase. Members of this family probably originated from a common ancestor from a primary metabolism pathway and have undergone substrate specialization leading to secondary metabolite pathways. Baker and Blasco (1992) suggested that this common ancestor could have been an UDP-galactose-4-epimerase involved in the modification of the sugar's C4 hydroxyl group. Weak homology (31%) was also found between VR-ERE and the HM1 gene encoding an NADPH-HC toxin reductase of maize. This enzyme is able to detoxify a cyclic tetrapeptide, called HC-toxin, produced by *Cochliobolus carbonum* (Johal and Briggs, 1992). The HM1 gene was the first cloned plant disease resistance gene. Interestingly, similarly to the VR-

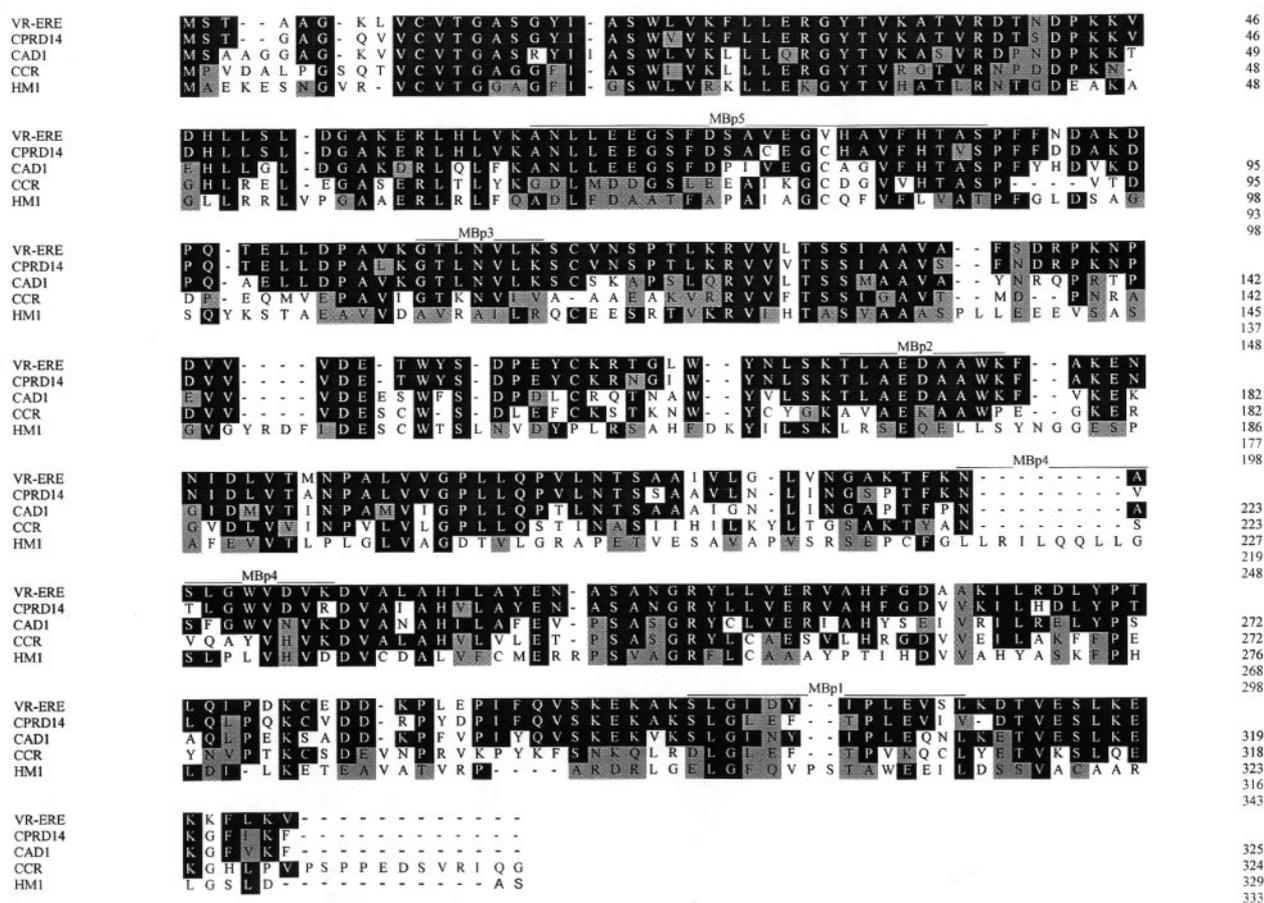


Figure 1. Multiple amino acid sequence comparison of *Vigna radiata* VR-ERE protein with proteins encoded by the *CPRD14* gene of *Vigna unguiculata* (accession number D83971), cinnamyl alcohol dehydrogenase (CAD1) from *Eucalyptus gunnii* (accession number X88797), cinnamoyl CoA reductase (CCR) from *Eucalyptus gunnii* (accession number X97433), and maize NADP(H): HC-toxin reductase (HMI) (accession number U77463). Sequences were aligned with the PC Gene program. Black and grey boxes contain residues which are identical and similar, respectively. Mb1 to Mb5 indicate the position of the fragments that have been sequenced after proteolysis of the purified *Vigna radiata* protein.

ERE gene, it is capable of converting a fungal toxin into an inactive metabolite. The N-terminal part was the most conserved among all these proteins (Figure 1) and includes a block of amino acids (11–38) corresponding to the putative NAD- and NADP(H) binding site (Perham *et al.*, 1991).

Functional expression of the VR-ERE cDNA in *E. coli*

The identity of the isolated *VR-ERE* cDNA was confirmed by expression of the recombinant protein VR-ERE in *Escherichia coli* (K38) in the pT7-7 expression vector system. The *E. coli* K38 (pT7-7/*VR-ERE*) lysate contained an over-expressed protein with an apparent molecular mass of 36 kDa, estimated after denaturing electrophoresis, which was in agreement with the size of the native *Vigna radiata* VR-ERE and the estimated molecular mass deduced from the nucleotide sequence. Protein extract of K38 cells harbouring the pT7-7/*VR-ERE* plasmid exhibited eutypine-reducing activity whereas no activity was found in control K38 cells (pT 7-7). K_m values for eutypine and NADPH

were 12.5 and 8.4 μM , respectively, and NADH was not an alternative cofactor. Thin layer chromatography analysis confirmed that eutypine was converted into the corresponding alcohol, eutypinol, in the presence of NADPH (data not shown).

Activity of the VR-ERE protein towards other aldehydic compounds

A variety of other linear and aromatic substrates containing an aldehyde function either present in plants (Table 2) or chemically synthesized (Table 3) have been found to be reduced by the VR-ERE enzyme. The kinetic properties of the enzyme in relation to naturally occurring aldehydes (Table 2) indicate that the enzyme always has a lower apparent K_m for eutypine than for any other compound. Furthermore, the catalytic efficiency (calculated as V_{max}/K_m) was about fourfold higher for eutypine than for the second most efficiently converted substrate, conyfaldehyde. The relative activity of the enzyme

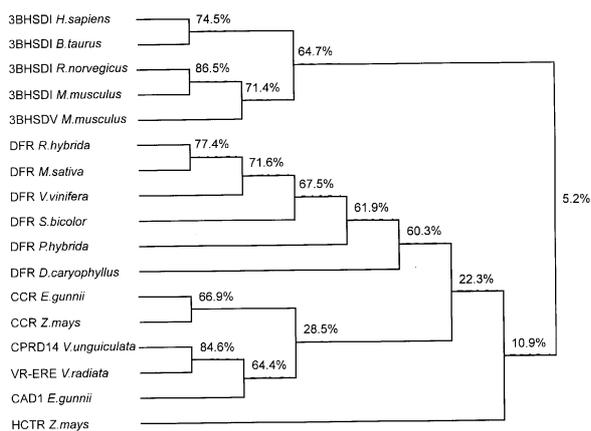


Figure 2. Dendrogram of full-length deduced amino acid sequences of VR-ERE and homologs including cinnamyl alcohol dehydrogenase (CAD), cinnamoyl CoA reductase (CCR), dihydroflavonol reductase (DFR) and 3- β -hydroxy-5-ene steroid dehydrogenase/ Δ -5 Δ -4 isomerase (3BHSD). The corresponding accession numbers are as follows: VR-ERE *V. radiata* (this study); CRDP14 *V. unguiculata* (D83971); CAD1 *E. gunnii* (X88797); CCR *E. gunnii* (X79566); CCR *Z. Mays* (X05068); DFR *R. hybrida* (X98083); DFR *M. sativa* (X80222); DFR *V. vinifera* (X75964); DFR *S. bicolor* (U87453); DFR *P. hybrida* (X15537); DFR *D. caryophyllus* (Z67983); 3BHSDI *H. sapiens* (M27137); 3BHSDI *B. taurus* (M58567); 3BHSDI *R. norvegicus* (M17614); 3BHSDI *M. musculus* (M58567); 3BHSDV *M. musculus* (L41519); HCTR *Z. mays* (U77463). The numbers correspond to the estimated percentage identities of the deduced amino acid sequences between two sublineages.

Table 2. Kinetic parameters of recombinant VR-ERE protein in relation to naturally occurring substrates. Activities were measured in the crude extract of *E. coli*

Substrates	$K_m(\text{app})$ μM	V_{max} $\text{nkatal mg}^{-1} \text{ protein}$	$V_{\text{max}}/K_m(\text{app})$
eutypine	12.5	3.22	$257.6 \cdot 10^{-3}$
benzaldehyde	45	0.79	$17.6 \cdot 10^{-3}$
salicylaldehyde	72	0.28	$3.9 \cdot 10^{-3}$
conyferaldehyde	83	5.19	$63.3 \cdot 10^{-3}$
sinapylaldehyde	117	3.08	$26.3 \cdot 10^{-3}$
cinnamaldehyde	128	0.86	$6.7 \cdot 10^{-3}$

Table 3. Aldehyde-reducing activity in the crude extract of *E. coli* transformed with the VR-ERE cDNA towards various synthetic aldehydic derivatives at 100 μM

Compounds	Relative activity	Compounds	Relative activity
eutypine	100	2-methoxybenzaldehyde	0
decylaldehyde	116 ± 24	3-methoxybenzaldehyde	166 ± 9
4-pyridine carbaldehyde	105 ± 7	4-methoxybenzaldehyde	116 ± 10
2-fluoroaldehyde	76 ± 6	2-nitrobenzaldehyde	86 ± 4
2-methylbenzaldehyde	0	3-nitrobenzaldehyde	170 ± 9
3-methylbenzaldehyde	141 ± 5	4-nitrobenzaldehyde	131 ± 9
4-methylbenzaldehyde	113 ± 3	2-fluorobenzaldehyde	140 ± 6
		3-fluorobenzaldehyde	148 ± 3
		4-fluorobenzaldehyde	115 ± 5

Activity towards eutypine (100%) corresponded to $0.31 \text{ nkatal mg}^{-1} \text{ protein}$. Control extracts from untransformed bacteria displayed no reductase activity towards any of the substrates. Values are means of three independent replicates \pm SE.

towards most of the substrates of chemical origin was in the same order of magnitude as towards eutypine, except for the 2-methyl and 2-methoxy forms of benzaldehyde which were not converted at all. Benzaldehyde derivatives, substituted at the 3-position, were better substrates than 4-substituted derivatives. Among the 3-substituted benzaldehydes, those harbouring voluminous groups were reduced at a higher rate (166% for methoxy and 170% for nitro) than those containing smaller groups (141% for methyl and 148% for fluoro). In our experimental conditions, no dehydrogenase activity had been detected when the VR-ERE protein was incubated in the presence of NADP and benzyl alcohol, suggesting that the reductase activity was essentially irreversible.

As could be expected given the homology of VR-ERE with enzymes of the lignin pathway, all three cinnamyl aldehyde precursors of lignins were found to be converted by the recombinant VR-ERE protein. However, coniferyl aldehyde was reduced at a higher rate (K_m 83 μM) than sinapyl aldehyde (K_m 117 μM) and cinnamaldehyde (K_m 128 μM) for which low but measurable reductions were detected. Benzaldehydes, therefore, were converted more efficiently by VR-ERE than any cinnamyl aldehyde derivatives. Despite sequence homology with enzymes of the flavonoid pathway, neither of the two major dihydroflavonols found in *Vigna radiata* (Ishikura *et al.*, 1981; Mato and Ishikura, 1993), namely dihydroquercetin and dihydromyricetin, were found to be substrates for VR-ERE. Therefore, the VR-ERE protein cannot be considered as either a CAD or a DFR, although well-conserved regions are shared by all three proteins (Figure 1). Among the closely related proteins CPRD14, CAD1 and VR-ERE, the substrate specificity of CPRD14 is unknown. CAD1 (Goffner *et al.*, 1998) and VR-ERE share strong affinity towards a broad range of aromatic aldehyde compounds. The biochemical characterization of CPRD14 would be useful to confirm the emergence of a new class of NADPH reductases exhibiting high

affinity for aromatic aldehydes in plants. The physiological function in the plant of this new class of enzymes remains to be established.

Two other genes capable of reducing aromatic aldehydes have been isolated from plants (aromatic NADP⁺ oxidoreductase, Somssich *et al.*, 1996) and bacteria (benzaldehyde alcohol dehydrogenase, Shaw *et al.*, 1992). However, their deduced amino acid sequences show no significant homology to VR-ERE (less than 6% at the amino acid level), indicating that divergent families of aromatic aldehyde reductases exist in living systems, although they may differ in their physiological functions.

Eutypine-reducing activity has been found in several plant species (*Vigna radiata*, *Daucus carota*, *Eucalyptus gunnii*) that are not a host for *Eutypa lata*. This is in contrast with the host-specific presence of the HM1 protein in the maize (Johal and Briggs, 1992). Evidence that enzymes expressed in living organisms are able to detoxify toxic compounds has been provided in bacteria and fungal organisms. An esterase, able to detoxify Brefeldin A, a host-selective toxin of the fungus *Alternaria caritham*, has been found in *Bacillus subtilis* (Kneusel *et al.*, 1994) and, recently, an albacidin (phytotoxin of *Xanthomonas albilineans*) detoxifying gene has been isolated from the bacterium *Pantoea dispersa* (Zhang and Birch, 1997). So far, the VR-ERE protein is the first example of a non-host-specific detoxification system of a pathogenic toxin in plants. Non-specific detoxification in plants has only been described for xenobiotics like herbicides such as atrazine or 2,4-D (Kreuz *et al.*, 1996). Their metabolism is generally achieved by constitutively expressed enzymes catalyzing various reactions such as oxidation, reduction and hydrolysis as, for example, cytochrome P450-dependent monooxygenase or glutathione-S-transferase. These detoxifying enzymes generally belong to the secondary metabolism of plants. In this respect, it is interesting that the VR-ERE protein also presents homology to enzymes of two secondary metabolic pathways, the lignin (CAD, CCR) and flavonoid (DFR) biosynthetic pathways.

Expression of the VR-ERE gene in *V. vinifera* cells cultured in vitro

The capacity of VR-ERE to confer resistance to the toxin eutypine was evaluated by over-expressing VR-ERE cDNA in *Vitis vinifera*. The cDNA was inserted in sense orientation between the 35S promoter and the terminator of octopine synthase of the binary expression vector PGA643 to create the construct pGA VR-ERE (Figure 3a). The construct was transferred into cells of a sensitive cultivar, Gamay, via *Agrobacterium*-mediated transformation. Southern blot analysis (Figure 3b) confirmed the integration of the VR-ERE construct into the genome of the three selected transgenic calli lines and indicated that

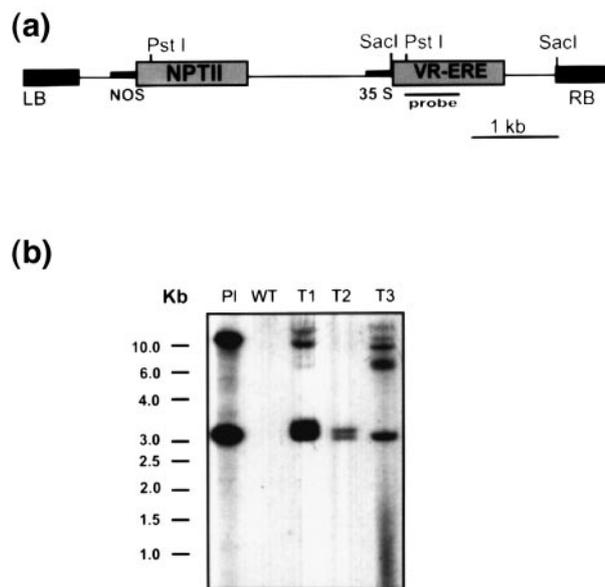


Figure 3. Molecular characterization of the transgenic lines of *Vitis vinifera* calli.

(a) Construction of the pGA binary vector plasmid that carries the VR-ERE cDNA driven by the CaMV 35S promoter, associated with the selectable marker gene, NPTII, under the control of the NOS promoter, and named pGA-VR-ERE.

(b) Southern blot analysis of 15 µg of genomic DNA isolated from wild-type calli (WT) and transgenic lines (T1-3). DNA was cut with *Pst*I and subjected to Southern blot hybridization using a 676 bp ³²P labeled cDNA insert corresponding to the 5' region of VR-ERE cDNA (44-720) as a probe. The positive control consisted of a plasmid pGA-VR-ERE, digested by *Pst*I. Positions of DNA fragment standards are indicated in kilobases.

the number of copies of the transgene varied from one to five. No hybridization was observed with DNA extracted from wild-type grapevine calli under these hybridizing conditions.

The three transgenic lines were then analyzed for VR-ERE expression and enzyme activity. A Northern blot analysis (Figure 4a) of total RNA showed that mRNAs hybridizing with the VR-ERE probe were present in the transgenic calli, but were undetectable in the non-transformed line, indicating that the transgene was efficiently transcribed. Western blot analysis (Figure 4b) was performed using polyclonal antibodies raised against the *Vigna radiata* VR-ERE recombinant protein. A strong immunoreactive signal was detected in the three independent transgenic calli, indicating a high accumulation of the VR-ERE protein in the transgenic cells. The three lines displayed eutypine-reducing activity ranging from seven- to 12-fold higher than the wild-type calli (Figure 4c). A good correlation was observed between amounts of mRNA, protein and activity level of VR-ERE for each transgenic callus. It was also observed that a 36 kDa protein present in a crude extract of untransformed calli showed weak cross-reaction with VR-ERE antibodies (Figure 4b) suggesting that a VR-ERE homolog protein was expressed constitutively in wild-

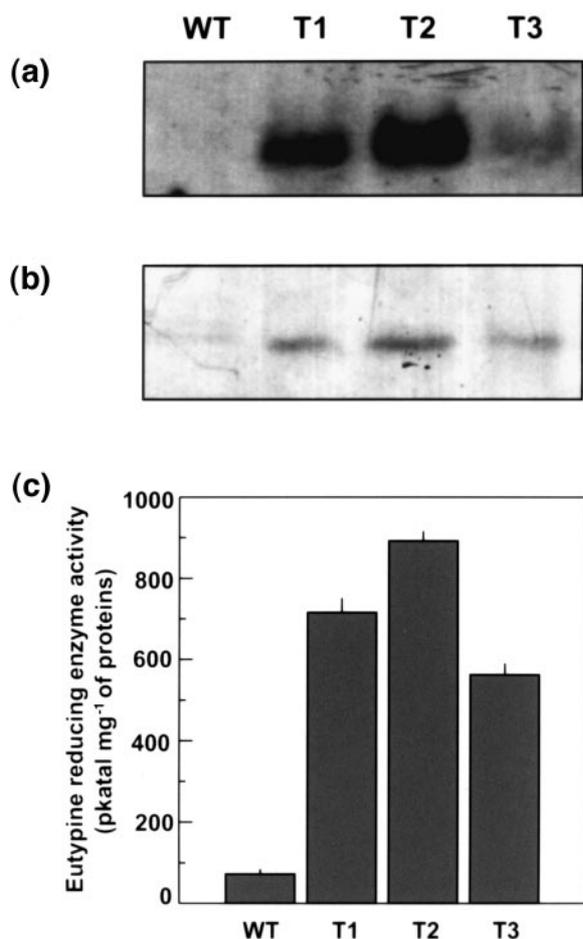


Figure 4. Expression of the *VR-ERE* cDNA in transgenic cell lines of *Vitis vinifera*.

(a) Northern blot of total RNA (20 μ g per lane), extracted from wild-type (WT) and transgenic calli (T1–3) and hybridized with *VR-ERE* cDNA as a probe.

(b) Western blot of total calli protein (15 μ g per lane) extracted from wild-type (WT) and transgenic calli (T1–3), separated in a 12% SDS polyacrylamide gel, immunoblotted and probed with purified antibodies against the recombinant *VR-ERE* protein.

(c) Eutypine-reducing enzyme activities in calli of wild-type (WT) and the *VR-ERE* transgenic lines T1–3. Values represent the means \pm SD from three separate extractions.

type *Vitis vinifera* Gamay cells and was partly or totally responsible for the low eutypine-reducing activity measured in wild-type calli.

In order to demonstrate that the over-expression of *VR-ERE* protein improves resistance of *Vitis vinifera* cells to the toxin, the wild-type and transgenic calli were grown under several concentrations of eutypine ranging from 0 to 1000 μ M (Figure 5). A eutypine concentration of 150 μ M was sufficient to reduce wild-type growth of calli by 50% compared to the eutypine-free medium. Growth of the transgenic calli was not affected by 500 μ M eutypine, while it was completely inhibited at the same concentration in wild-type *Vitis vinifera* cells (Figure 6). Interestingly, all three independent calli, although exhibiting different

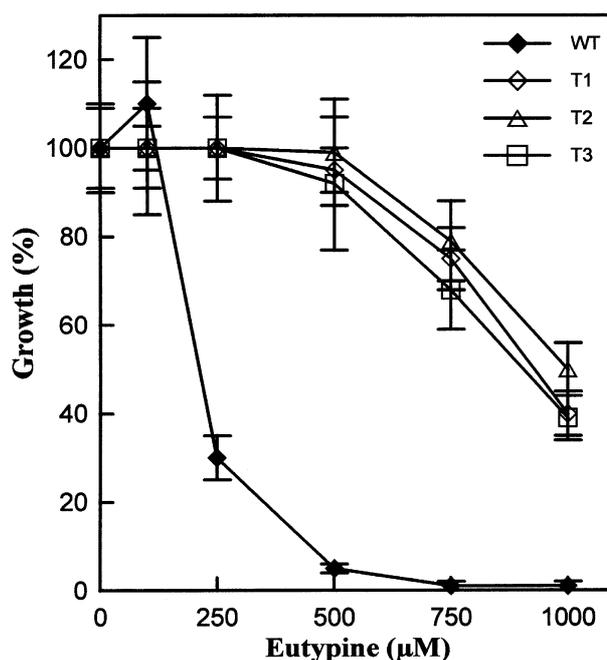


Figure 5. Resistance to eutypine of *VR-ERE* transgenic lines of *Vitis vinifera*, cv. Gamay, callus in culture.

Callus of wild-type (WT) and transformed lines (T1, T2, T3) were cultured in the presence of a range of eutypine concentrations. Callus weight was determined after 4 weeks in culture. Each point represents a mean of 40 replicates.

eutypine-reducing activities, displayed similar resistance to eutypine.

In conclusion, the present data clearly demonstrate that the *VR-ERE* gene is able to confer resistance of *Vitis vinifera* cells to the toxin eutypine. It remains to be determined whether the over-expression of the *VR-ERE* gene will confer tolerance or insensitivity of whole grapevine plants to *Eutypa lata*. For this purpose, transgenic *Vitis vinifera* plants expressing the *VR-ERE* gene are currently being engineered. As *Eutypa lata* also induces symptoms on other woody species such as apricot or cherry trees (Munkvold and Marois, 1991), this strategy has the potential to be extended to these plants.

Experimental procedures

Chemicals

Benzaldehyde and benzyl alcohol were purchased from Sigma (Chemical Company, St Louis, MI, USA), cinnamaldehyde from Fluka (Chemika-Biochemika, Buchs, Switzerland), and all other chemicals from Aldrich (Chemie, Steinheim, Germany). [¹⁴C] eutypine was synthesized according to the method of Defrancq and Tabacchi (1992).

Plant material

Seeds of *Vigna radiata* L. R. Wilcz were purchased from Cereal Wander Nutrition Annonay, France. Callus and cell suspension



Figure 6. Eutypine resistance displayed by transgenic grapevine calli (cv. Gamay) cultured *in vitro* (line T2) expressing a cDNA encoding a eutypine-reducing enzyme of *Vigna radiata*.

The photograph was taken after 20 days of culture.

cultures of *Vitis vinifera* cv. Gamay were grown using the protocol of Ambid *et al.* (1983).

Eutypine-reducing enzyme activity assay

The eutypine-reducing enzymatic activity was assayed spectrophotometrically at 25°C by measuring the rate of the enzyme-dependent decrease of NADPH absorption at 340 nm. The reaction mixture contained 25 mM Tris-HCl (pH 7.0), 100 μM NADPH and 100 μM eutypine or other aldehyde derivatives. K_m values were determined from initial reaction velocities by varying the substrate and cofactor concentrations between 6.25–100 μM. Kinetic parameters were calculated assuming Michaelis-Menten type performance of the enzyme.

Purification and peptide sequencing of VR-ERE from V. radiata

Etiolated mung bean hypocotyls were homogenized in two volumes (v/w) of extraction medium (0.1 M borate buffer (pH 8.0), 10% (w/v) glycerol, 1% (v/v) polyvinylpyrrolidone (M_w 40 000) and 4 mM DTT) and centrifuged for 20 min at 48 000 *g*. After centrifugation, proteins in the supernatant were precipitated using ammonium sulfate. The proteins precipitating between 30% and 70% saturation were redissolved in a small volume of buffer A (25 mM K-phosphate buffer (pH 8.0), 10% glycerol, 1 M (NH₄)₂SO₄) before loading onto a 16 mm × 300 mm Phenyl-Sepharose CL-4B (Pharmacia, Saclay, France) previously equilibrated with buffer A. Fractions exhibiting eutypine-reducing activity in the assay described above were pooled and loaded on a hydroxyapatite column (Econo-Pac HTP Cartridge, Bio Rad, Hercules, USA) equilibrated with 10 mM K-phosphate (pH 8.0), 10% glycerol. Fractions showing eutypine-reducing activity were again pooled and

concentrated to a volume of 300 μl by ultrafiltration using a Centriscart C4 membrane (Sartorius, Palaiseau, France). The concentrated samples were subjected to liquid chromatography (Waters 650E) using a 300 mm × 10 mm (internal diameter) Superose 12 HR column (Pharmacia, Saclay, France) equilibrated with 25 mM K-phosphate (pH 8.0), 10% glycerol. Active fractions were subjected to further chromatography using an FPLC MonoQ HR-5/5 column (Pharmacia, Saclay, France) equilibrated with 25 mM Tris-HCl (pH 8.0), 10% glycerol. The final step consisted of separating a 36 kDa polypeptide using SDS PAGE, followed by electroelution of the desired protein. After digestion with an endoproteinase Lys-C (Boehringer Mannheim, Meylan, France), the resulting peptides were separated by reverse-phase HPLC. Five of the peptides were sequenced by Edman degradation using an Applied Biosystems 470 amino acid microsequencer (Institut Pasteur, France). The sequences obtained, MBp1, MBp2, MBp3, MBp4 and MBp5, are given in Table 1. Two degenerate primers, namely MBn1 and MBn5, were designed from these sequences (Table 1).

Preparation and isolation of cDNA for VR-ERE from Vigna radiata

Poly(A)⁺ RNA was isolated from *V. radiata* hypocotyls using the PolyAtract system (Promega, Charbonnières, France). First and second strand cDNA synthesis and the addition of the adaptors were performed using proprietary kits (Pharmacia, Saclay, France) according to the manufacturer's instructions. cDNA, at a final concentration of 2 ng μl⁻¹, was self-ligated overnight at 12°C. The resulting products were used as a template for PCR amplification of the VR-ERE cDNA fragment between the oligonucleotides MBn1 and MBn5. The amplification was performed with an initial denaturation at 95°C for 3 min, followed by 30 amplification cycles

of 95°C for 1 min, 52°C for 1 min and 72°C for 2 min. The 5' and 3' cDNA regions were amplified by inverse PCR (iPCR) which was performed on circularized cDNA using the two primers, Mbi5' reverse (GGCATCGTTGAAAAGGGAG) and Mbi3' forward (CGAAGGAAAAAGCAAACAGC). The full-length cDNA was finally amplified using two primers located in the ends of both 5' (TCAACATAATCCACAATGAGCAC) and 3' (GATAAAAACATAATAATGGGTGC) regions using Pfu polymerase (Stratagene, Heidelberg, Germany). Three independent clones were sequenced by the dideoxynucleotide chain termination method (Sanger *et al.*, 1977) using a Sequenase kit (United States Biochemical).

The amino acid sequence corresponding to the nucleotide sequence of the cDNA was predicted using the TRANSL program of the PC Gene software package (Department of Medical Biochemistry, University of Geneva, Switzerland). A computer search for protein similarities was performed with the BLAST program (Altschul *et al.*, 1990) using Genpept, PIR and Swissprot databanks. Multiple protein sequence alignments were performed using the CLUSTAL program (Thompson *et al.*, 1994) of the PC Gene software package.

Expression in *E. coli*

The coding region of the *VR-ERE* cDNA was directly cloned into the *EcoRI* site of the expression vector, pT7-7, of Tabor and Richardson (1985). Expression of the recombinant protein was induced by incubating bacterial cultures at 42°C for 20 min. After a further culture period of 30 min at 37°C, bacteria were lysed by sonication in 20 mM Tris-HCl buffer (pH 7.5) containing 1 mM EDTA, 10% glycerol (w/v), 0.1% NP 40 (w/v), 1 mM PMSF, 5 mM DTT, and centrifuged at 10 000 *g* for 5 min.

Preparation of antiserum against recombinant *VR-ERE* protein and Western blotting

Recombinant protein was purified by preparative SDS-PAGE (12% polyacrylamide gel) (Laemmli, 1970) and electroelution (Electroeluter model 422, Bio-Rad, Hercules, USA) according to the manufacturer's instruction. Polyclonal antibodies were produced in rabbits (Eurogentec, Seraing, Belgium) and partially purified using a 0.45 µm nitrocellulose membrane (Bio Rad, Hercules, USA) onto which a crude protein extract of wild-type *E. coli* had been fixed. Proteins from *Vitis vinifera* cells were extracted in 100 mM Tris-HCl buffer (pH 7.5), 2.5 mM DTT, 2.5% polyvinylpyrrolidone and centrifuged at 10 000 *g* for 10 min, then used for Western blotting and eutypine-reducing enzyme activity assays. Proteins (15 µg) were separated in 12% polyacrylamide gels according to Laemmli (1970) and transferred to nitrocellulose membranes (Trans-Blot Transfer Medium, Bio Rad, Hercules, California) as described by Towbin *et al.* (1979). The membranes were probed with purified antibodies (1:2000 dilution in 20 mM Tris-HCl buffer (pH 7.6), 137 mM NaCl, 1% gelatine) for 1 h, then incubated for 1 h with antirabbit IgG-conjugated peroxidase according to the manufacturer's recommendations (Sigma, Saint Quentin Fallavier, France). The protein-antibody complex was detected using the ECL Western blotting detection system (Amersham, Les Ulis, France).

Northern and Southern analyses

Genomic DNA and total RNA were isolated from *Vitis vinifera* cells grown *in vitro* according to the method of Doyle and Doyle

(1990) and Wadsworth *et al.* (1988), respectively. Total RNA and *PstI* or *SacI* digested genomic DNA were separated in either 1% formaldehyde/agarose (RNA) or 0.8% agarose (DNA) gels, and then transferred to GeneScreen Plus membranes according to the manufacturer's instructions (Dupont de Nemours). Northern and Southern hybridizations were performed overnight in 2 × SSC, 5 × Denhardt's, 1% SDS, 20 µg ml⁻¹ salmon sperm DNA (Sambrook *et al.*, 1989) at 60°C. A 676 bp cDNA fragment corresponding to the 5' region of *VR-ERE* cDNA (44–720) was labelled using a random priming kit (Amersham, Les Ulis, France) and used as a probe. After hybridization, membranes were washed at 60°C, twice in 0.1 × SSC, 0.1% SDS for 15 min for Southern blots and in 0.2 × SSC, 0.1% SDS for 15 min for Northern blots, and then exposed to X-ray film using intensifying screens at –80°C.

Transformation of *Vitis vinifera* cells cultured *in vitro*

Transformation was performed using 10-day-old callus cultures using *Agrobacterium tumefaciens*, strain C58, containing the binary vector pGA643 (An *et al.*, 1988) with a Nos/NPTII chimeric gene and the *VR-ERE* cDNA under the transcriptional control of the *CaMV* 35S promoter (Figure 4a). Calli of *Vitis vinifera* cv. Gamay were submerged for 5 min in an overnight culture of *A. tumefaciens* (10⁶–10⁸ cells ml⁻¹), dry blotted, then co-cultivated on growth medium (Ambid *et al.*, 1983) in the dark for 48 h at 28°C. Finally, transformed cells were selected on growth medium containing 100 µg ml⁻¹ kanamycin and 500 µg ml⁻¹ carbenicillin.

Acknowledgements

This work was supported by EEC program N°. 8001-CT91.205, the French 'Ministère de l'Enseignement Supérieur et de la Recherche' and Midi-Pyrénées Region. P.G. and G.R.M were recipients of an EU fellowship. M.G and S.C. hold grants from the 'Ministère de l'Enseignement Supérieur et de la Recherche'.

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