Constitutive expression of *Vr-ERE* gene in transformed grapevines confers enhanced resistance to eutypine, a toxin from *Eutypa lata*

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Abstract

Eutypine (4-hydroxy-3-[3-methyl-3-butene-1-ynyl] benzaldehyde) is a toxin produced by *Eutypa lata*, the causal agent of eutypa dieback of grapevines. Recently, a eutypine detoxifying gene (*Vr-ERE*) encoding an NADPH-dependent aldehyde reductase which converts eutypine into the corresponding alcohol, eutypinol, a non-toxic form of the toxin, has been cloned. We report here the obtention of transgenic plants from grapevine rootstock 110 Richter *Vitis berlandieri* × *V. rupestris* that express the *Vr-ERE* gene. The over-expression of *Vr-ERE* gene in grapevine tissues increases their detoxification capacity. The growth and development of the transgenic plants cultured in vitro exhibiting a high Vr-ERE activity were not affected by the presence of 300 μM of toxin whereas the growth of untransformed control plants was highly inhibited. These results suggest that the *Vr-ERE* gene is an efficient candidate to confer resistance to the toxin and opens new opportunities to study the role of the toxin in the development of the disease.

Keywords: *Eutypa lata*; Eutypa dieback; Eutypine; Toxin; Detoxification; Reductase; Grapevine; Genetic transformation

1. Introduction

Many pathogenic fungi produce phytotoxins that induce characteristic symptoms in their plant hosts. The degree of toxin production is commonly associated with disease severity and can permit colonization or systemic invasion by the pathogen [1]. In most cases, it has been shown that toxin resistance is based on the ability of the plants to detoxify the pathogenic toxins [2–4]. Various mechanisms of disease resistance have been studied in recent years using genetic modification of plants to enhance resistance to the toxins involved in the development of the disease [5,6].

Eutypa dieback, caused by the ascomycete fungus *Eutypa lata* (Pers.: Fr.) Tul., is one of the most devastating grapevine diseases in many countries, is responsible for considerable losses of yield and has a very important economic impact [7,8]. The causal agent infects the vine stock through pruning wounds, invades the xylem and phloem of the trunk and branches and forms a canker around the infected wound [9,10]. After a long incubation period of 3 years or more, the symptoms of eutypa dieback in the herbaceous parts of the plant lead to dwarfed and withered new growth of the branches, marginal necrosis of the leaves, dryness of the inflorescence, and death of one or more branches [11]. The pathogen produces a toxin, named eutypine (4-hydroxy-3-[3-methyl-3-butene-1-ynyl] benzaldehyde) that is transported by the sap to the herbaceous part of the plant [12–14]. The toxin appears to be an important virulence factor involved in symptom development of the disease [15]. Eutypine penetrates grape-

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vine cells through passive diffusion and exhibits a protonophoric activity, in particular uncoupling mitochondrial oxidative phosphorylation [16,17].

Eutypine has been found to be converted by grapevine tissues into a compound identified as eutypinol (4-hydroxy-3-[3-methyl-3-butene-1-ynyl]benzyl alcohol) that is not toxic for grapevine tissues [18]. This detoxification mechanism plays a role in defense reactions but it has not been characterized in grapevines tissues. Recently, a mechanism of eutypine detoxification has been identified in *Vigna radiata* through the enzymatic reduction of eutypine into its corresponding alcohol, eutypinol. A gene, named *Vr-ERE* , has been cloned in *V. radiata* and encodes an NADPH-dependent aldehyde reductase of 36 kDa which is able to reduce eutypine into eutypinol [19]. This enzyme exhibits a high affinity for the toxin.

However, the long incubation period of the fungus before any symptoms of the disease become apparent, and the absence of toxin-deficient mutants of the fungus have prevented critical study of the toxin in grapevine plants. The availability of a eutypine resistance gene is of great interest as a critical tool to assess the role of eutypine in the development of eutypa dieback disease. As a prerequisite to demonstrate the role of eutypine in symptom development and in the invasion of the pathogenic fungus through the plant, the *Vr-ERE* gene has been introduced in the grapevine by genetic engineering. We report here that expression of *Vr-ERE* gene greatly increases the detoxification capacity of transgenic grapevine tissues and can confer enhanced resistance to eutypine.

2. Materials and methods

2.1. Plant material and transformation

Somatic embryogenic callus culture obtained from the vegetative tissues of anthers of the grapevine 110 Richter *V. berlandieri* × *V. rupestris* by Bouquet et al. [20] was used. Embryogenic calli were cultured in a Petri dish (55 mm) on NB medium [21] containing half-strength MS medium salts [22], gelified with 0.7% (w/v) agar and supplemented with 3% (w/v) sucrose, 5 μM naphthoxyacetic acid (NOA) and 1 μM benzyladenine (BA). The cultures were incubated in the dark at 26 ± 0.5 °C.

Transformation was performed using disarmed *Agrobacterium tumefaciens*, strain C58, containing the binary vector pGA643 [23] harbouring a *nos*/*npt*II chimeric gene and the *Vr-ERE* cDNA under the transcriptional control of the *CaMV* 35S promoter according to the method described by Le Gall et al. [21]. Briefly, 2 weeks before inoculation, embryogenic calli were transferred from NB medium to hormone-free NB medium. Calli were inoculated with a bacterial suspension and incubated for 30 min, at 26 ± 0.5 °C, in the dark. They were then blotted dry with sterile paper and cultured on the NB medium in the dark for 48 h, at 28 °C. After the co-cultivation period, the calli were washed three times with liquid NB medium containing 500 s^{-1} m^{-2} carbenicillin and transferred to a selective NB medium containing 500 s^{-1} m^{-2} carbenicillin and 4 s^{-1} m^{-2} kanamycin. Embryogenic calli were sub-cultured every 4 weeks on the fresh NB medium containing the same concentrations of carbenicillin and kanamycin.

After 3 months or more, the calli gave well-developed somatic embryos that were then transferred to the growth medium consisting of hormone-free NB medium containing 4 s^{-1} m^{-2} kanamycin and cultured at 26 ± 0.5 °C, under 16 h light/8 h dark cycle with a light flux of 100 μmol s^{-1} m^{-2}. Each regenerated somatic embryo constituted a clone that was further micropropagated by microcuttings in a Magenta vessel containing 80 ml of plant growth regulator-free NB medium and 4 s^{-1} m^{-2} kanamycin. When the microcuttings developed roots then a shoot within 3 weeks, the grapevine lines were considered as putatively transformed and retained for further investigations. Morphological observations, biochemical and molecular studies were performed on in vitro cultured grapevine plants.

2.2. PCR analysis

Kanamycin-resistant grapevine transformants were tested for the presence of *Vr-ERE* cDNA by PCR. Genomic DNA was extracted from leaves according to Le Gall et al. [21]. DNA was resuspended in 10 mM Tris–HCl, pH 8.0. PCR reactions were performed in 50 μl volumes containing 10 mM Tris–HCl, pH 8.0, 50 mM KCl, 1.5 mM $MgCl_2$, 10 μM dNTPs, 0.5 U of Taq polymerase, 0.4 μM oligonucleotide primers and 50 ng of plant DNA. Amplification of the *Vr-ERE* cDNA sequence was performed with 5′ (5′-GTCTGTGTCAC-CGGCGCTTCC-3′) and 3′ (5′-TGCTAGAATATGG-GCCAATCA AC-3′) sequences as primers, using a Stratagene RCG40 thermocycler with 30 cycles of denaturation (95 °C for 45 s), annealing (68 °C for 1 min) and extension (72 °C for 1 min) with a 5 min extension at 72 °C for the last cycle.

2.3. Extraction and gel-blot analysis of DNA, RNA and protein

Genomic DNA was extracted from grapevine leaves excised from in vitro plantlets according the procedure described for PCR analysis. Then, *Sac*I and *Hind*III digested DNA was separated in 0.7% agarose gel, and transferred to GeneScreen Plus membranes according to the manufacturer's procedure (Dupont de Nemours, Switzerland). Hybridizations were performed overnight in 6 × SSPE, 5 × Denardt's, 0.5% SDS, 50% formamide,

100 μg ml^{-1} salmon sperm DNA as described by Sambrook et al. [24] at 42 °C. A 696-bp cDNA fragment corresponding to the 5′ region of *Vr-ERE* cDNA (44–740) was labelled using a random probing kit (Amersham, UK) and used as a probe. After hybridization, membranes were washed at 42 °C, twice in 2 × SSC, 0.1% SDS for 10 min, and then exposed to X-ray film using intensifying screens at −80 °C.

Total RNA was extracted according to Boss et al. [25] from leaves excised from in vitro plantlets and frozen in liquid nitrogen. Total RNA was separated in 1.2% formaldehyde/agarose and then transferred onto GeneScreen Plus membranes, and cross-linked with a UV crosslinker (Amersham, UK). Hybridization and washing conditions as well as the probe were the same as described above. Northern RNA quantification was achieved by using an 18S-rRNA probe. This experiment was repeated with different RNA extracts with the same quantity of RNA and similar patterns were obtained for each analysis.

Proteins (15 μg), extracted as described for ERE assays, were separated in 12% acrylamide gels according to Laemmli [26] and transferred to nitrocellulose membranes (Trans-Blot Transfer Medium, Bio Rad, Hercules, CA) as described by Towbin et al. [27]. 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, St. Louis, MO). The protein antibody complex was detected using an ECL Western blotting detection system (Amersham, Les Ulis, France).

2.4. Eutypine reductase enzyme (ERE) assays

Proteins from leaves of the in vitro plants were extracted in 100 mM Tris–HCl buffer (pH 7.5), 2.5 mM DTT, 2.5% polyvinylpolypyrrolidone and centrifuged at 10 000 × *g* for 10 min, then used for eutypine-reducing enzyme assays. ERE activity was assayed spectrophotometrically at 25 °C by measuring the rate of enzyme-dependent decrease of NADPH absorption at 340 nm as described previously [18]. Briefly, the reaction mixture consisted of 200 mM Na_2HPO_4/100 mM citric acid, pH 6.5, 100 μM NADPH, 100 μM eutypine and 100 μl of protein extracts. ERE assays were performed in triplicate from two independent extracts of each transformed clone. Protein content was determined using a bicinchoninic acid dye reagent (Pierce) and BSA as a standard. To verify the identity of the reaction products, eutypine was substituted by [^{14}C]eutypine, then the samples were analysed by chromatography as described by Colrat et al. [18].

2.5. Assessment of resistance to eutypine

Microcuttings of transgenic and untransformed grapevine plants were collected from 2-month-old in vitro plants, then cultured in regulator-free NB plant growth medium in the presence of various concentrations of eutypine ranging from 0 to 400 μM. The growth of the plants was determined after 3 months of culture by measuring the stem length. For each experiment, 20 microcuttings were cultured and the results are presented as the mean of three independent experiments.

3. Results and discussion

3.1. Analysis of transgenic plants constitutively expressing the Vr-ERE gene

Somatic embryogenic callus of grapevine were co-cultivated with *A. tumefaciens*, strain C58, containing the binary vector pGA643 harbouring the *Vr-ERE* gene under the control of the *CaMV* 35S promoter and the NPTII gene (Fig. 1A). Transformants were selected on the basis of their capacity to grow in the presence of kanamycin and the presence of *Vr-ERE* cDNA assessed by a polymerase chain reaction-based method (data not shown). Among 20 kanamycin-resistant regenerated plants, three transformed lines (CL7, CL12 and CL41) were further studied.

Integration of the transgene in these three transgenic lines was confirmed at the genomic level by DNA gel-blot analysis. The grapevine plants of the three selected lines incorporated the *Vr-ERE* gene (Fig. 1). As expected from the restriction map of the T-DNA insert, digestion of DNA with *Sac*I yielded a 1.8-kb fragment diagnostic for the transgene that was visible in the DNA of the three transgenic lines but not in untransformed plant DNA, following hybridization with the *Vr-ERE* cDNA probe (Fig. 1B). After probing of genomic DNA digested with *Hind*III which cuts the transformation plasmid once on the 5′ side of the construct, one fragment was observed in the CL7 and CL41 lines and four fragments in the CL12 plant, suggesting that several copies of the transgene had been inserted in this line (Fig. 1C).

3.2. Expression of the Vr-ERE gene in grapevine plants

Total RNA was extracted from leaves of 2-month-old plants cultivated in vitro and analysed by RNA gel blot hybridization with *Vr-ERE* cDNA as a probe. The transgenic CL7, CL12 and CL41 plants constitutively expressed the *Vr-ERE* transgene whereas the untransformed plants showed no hybridization with the *Vr-ERE* cDNA as a probe (Fig. 2A). Furthermore, Western blot analysis of proteins, extracted from leaves of the

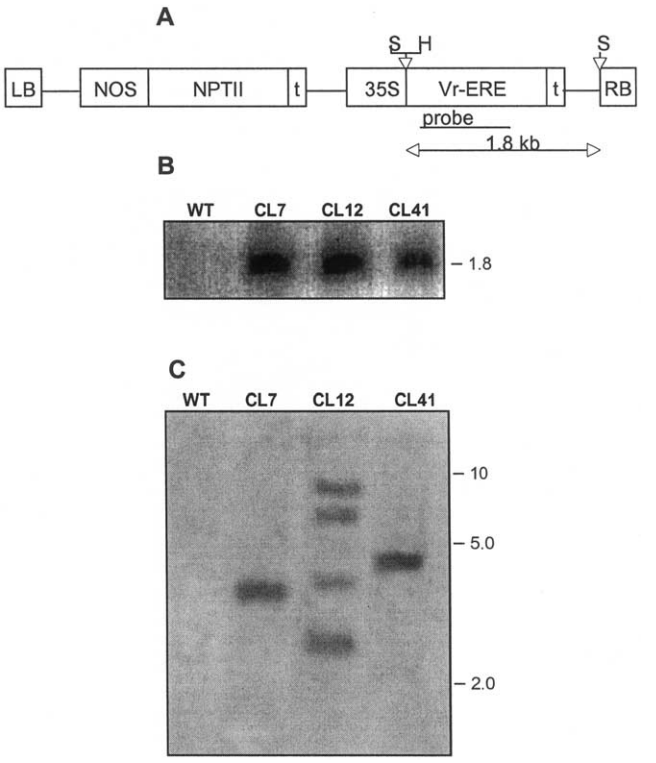

Fig. 1. Schematic structure of transformation vector and analysis of transgenic plants. (A) Construction of the pGA–VR-ERE binary vector plasmid harbouring the *VR-ERE* cDNA driven by the *CaMV* 35S promoter, associated with the selectable marker gene, *NPTII*, under the control of the NOS promoter. H: *Hind*III; S: *Sac*I. All *Hind*III and *Sac*I sites present in pGA–VR-ERE are indicated. (B) and (C) DNA gel blot analysis of untransformed control plants (WT) and transformed lines CL7, CL12 and CL41. Genomic DNA was digested with *Sac*I (B) which cuts twice within the construct, or with *Hind*III (C) which cuts the plasmid once at the 5′ side of the construct, and hybridised with a radiolabelled 696-bp cDNA probe. Numbers on the right indicate the size of the marker bands in kilobases.

three transformed lines, using antibodies raised against fusion protein, revealed a 36-kDa protein corresponding to the Vr-ERE protein accumulated in transgenic plants but not in untransformed control plants (Fig. 2B).

Eutypine reductase activity was determined using protein extracts from leaves excised from untransformed and transformed plants. A significant increase in eutypine reductase activity was found in the plants of transformed lines as compared to those of the untransformed control plants (Fig. 3). The data suggest a correlation between the amounts of RNA and protein, and the enhancement of ERE activity for each of the transgenic lines.

In addition, the results show that leaves of untransformed plants are also able to reduce eutypine into eutypinol. This endogenous detoxification mechanism cannot be attributed to the expression of a homologous *Vr-ERE* gene since no hybridization was observed with DNA extracted from wild-type grapevine plants and no immunoreactive signal with antibodies raised against the

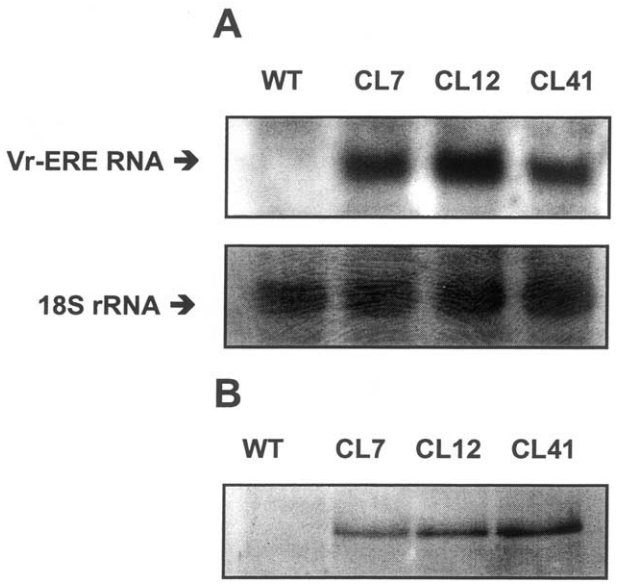

Fig. 2. Analysis of *Vr-ERE* expression in transgenic plants. (A) RNA gel blot analysis of CL7, CL12 and CL41 transgenic plants and untransformed control plants (WT). Total RNA was extracted from leaves and aliquots (20 μg) were fractioned using denaturing gel and hybridized with a radiolabelled *Vr-ERE* cDNA probe. RNA was quantified by hybridization using 18S-rRNA as a probe. (B) Protein gel-blot analysis of CL7, CL12, CL41 and control (WT) plants. Proteins extracted from in vitro plant leaves were separated (15 μg per lane) in a 12% SDS polyacrylamide gel. The blot was probed with a rabbit antiserum raised against the Vr-ERE fusion protein and processed with an ECL chemiluminescence detection system.

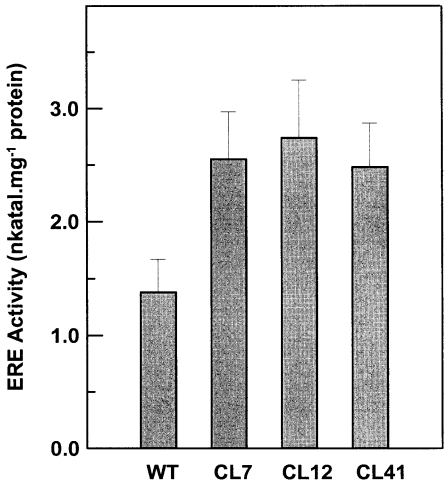

Fig. 3. Eutypine-reducing enzyme activities in leaves of transgenic lines CL7, CL12, CL41 and control (WT) plants. Values represent the means ± S.D. from three separate extractions.

Vr-ERE fusion protein was detected under our hybridisation conditions. These data suggest that grapevine constitutively expresses one or several proteins exhibiting an NADPH-dependent eutypine reductase activity. These proteins are able to catalyse the reduction of eutypine but seem to be different from Vr-ERE since no

hybridization was observed with proteins extracted from wild-type grapevine. They contribute to endogenous detoxification by inactivating eutypine. Previously, a 54-kDa reductase protein involved in the enzymatic degradation of eutypine was described in *Vitis vinifera* cv. Gamay cells, cultured in vitro [18]. It cannot be excluded that such an aldehyde reductase is involved in detoxification of eutypine in the 110 Richter cultivar.

3.3. Effects of Vr-ERE gene expression on resistance to eutypine

It has been demonstrated that eutypine uncouples mitochondrial oxidative phosphorylation via a cyclic protonophore mechanism and in vitro inhibits grapevine growth [17]. In order to evaluate the effects of *Vr-ERE* gene expression on the resistance to eutypine, microcuttings of untransformed control plants and transformed plants were cultured in the presence of various concentrations of eutypine. The growth of the vines, expressed as stem length, was scored after 2 months of culture. The results of the three independent experiments show that the growth of the untransformed plant was greatly inhibited (90%) in the presence of 300 μM eutypine whereas that of transformed plants was less strongly reduced (20–35%) (Fig. 4). The 50% inhibition of growth was reached for a eutypine concentration of 230 μM for the control plant whereas it required 375 μM eutypine for the CL12 line. In the cells of the untransformed plants, when the eutypine concentration reached a critical threshold level, the endogenous detoxification mechanism was not sufficient to inactivate the toxin accumulated in the tissues. Thus, its toxicity, in particular affecting the cell membrane, can inhibit growth [16–28]. In contrast, in transgenic lines, the expression of the *Vr-ERE* gene significantly enhanced resistance to eutypine by increasing the detoxification capacity of the cells.

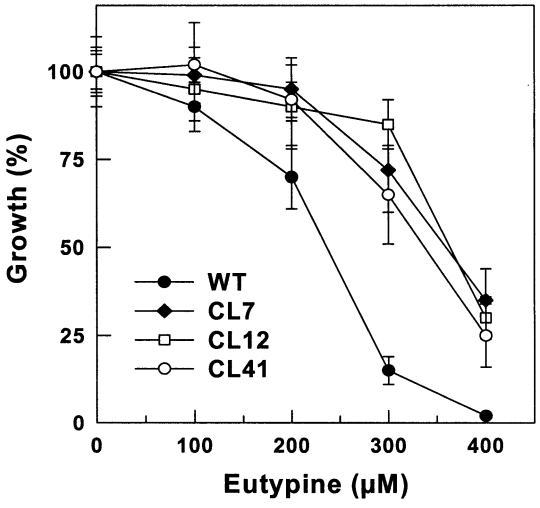

Fig. 4. Enhanced resistance of transgenic plants against the toxin eutypine. Microcuttings from untransformed control plants (WT) and transformed lines (CL7, CL12, CL41) were cultured in the presence of a range of eutypine concentrations. The stem length of the plants was measured after 8 weeks of culture. Each point represents the means ± S.D. of three independent experiments.

In conclusion, the results presented here show that *Vr-ERE* gene expression enhances the detoxification capacity of eutypine and contributes to increasing the resistance of grapevine plants to the toxin. The morphology of transgenic plants cultured in vitro was apparently unaffected by the expression of the *Vr-ERE* gene. The availability of transgenic clones with an appropriate pattern of *Vr-ERE* gene expression is therefore a promising approach to understand the role of eutypine in the development of disease symptoms and in the multiplication of the toxigenic pathogen in the grapevine plant. It remains to be determined if the overexpression of the *Vr-ERE* gene will confer tolerance or insensitivity to *Eutypa lata* in grapevine plants. For this purpose, transgenic grapevine plants will be acclimatized and cultivated in the greenhouse and after a development period of 2–3 years, lignified cuttings could be infected by pathogenic fungus as previously described by Péros et al. [29], in order to assess the role of eutypine in fungus colonisation and in the development of the disease symptoms.

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