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Title

Effect of ectopic expression of the eutypine detoxifying gene *Vr-ERE* in transgenic apple plants

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Keywords aldehyde reductase, apple, eutypine, genetic engineering, *Malus x domestica*

Abstract

Development of alternative selection systems without antibiotic resistance genes is a key issue to produce safer and more acceptable transgenic plants. Eutypine is a toxin produced by *Eutypa lata*, the causal agent of eutypa dieback of grapevine, which is detoxified in mung bean (*Vigna radiata*) by the gene (*Vr-ERE*). Many phytotoxic compounds containing an aldehyde group can act as substrates for the Vr-ERE enzyme. The aim of the present work was to evaluate the effects of the overexpression of *Vr-ERE* in transgenic apple plants, as a first step towards the development of an alternative selection system. Viable transgenic apple clones expressing *Vr-ERE* were produced from the cultivar Greensleeves under kanamycin selection. Although the *Vr-ERE* transgene was normally expressed at RNA and protein levels, the increase in aldehyde reductase activity tested on a range of potential substrates was very low in these clones. None of them revealed a significant increase in tolerance to toxic aldehydes compared to their non-transgenic control. This work with transgenic apple plants overexpressing the detoxifying gene *Vr-ERE* illustrates some of the difficulties in developing an alternative selection pressure.

Abbreviations BA : 6-Benzyladenine BD: benzaldehyde CaMV :Cauliflower mosaic virus Conyl: coniferylaldehyde Decyl: decylaldehyde 3FBD: 3-fluoro-benzaldehyde GUS : β -Glucuronidase IBA :Indole-3-butyric acid 2HBD: 2-hydroxy-benzaldehyde 3HBD: 3-hydroxy-benzaldehyde 4HBD: 4-hydroxy-benzaldehyde 3MBD: 3-methoxy-benzaldehyde 4MBD: 4-methoxy-benzaldehyde MS: Murashige and Skoog 3NBD: 3-nitro-benzaldehyde

4NBD: 4-nitro-benzaldehyde, 4PBD: 4-pyridoxy-benzaldehyde TDZ: Thidiazuron Tolyal:
tolylaldehyde

INTRODUCTION

Current apple transformation methods are based on the use of selectable marker genes that confer resistance to antibiotics as a selection pressure (Brown and Maloney 2005). However, public concern about food safety of genetically engineered plants and potential horizontal flow of antibiotic resistance genes requires new alternative strategies. A few reports have indicated the feasibility of alternative selection pressure for apple transformation. The bialaphos resistance gene (*bar*) enabled the production of transgenic apple using 1 mg/l bialaphos as a selection pressure in *Malus prunifolia* Asami (Ogasawara et al., 1994) and in Elstar and Holsteiner Cox (Szankowski et al., 2003). Even though several *bar*-containing crop plants have received approval for sale, the use of an herbicide-resistance marker does not answer all the public concerns about environmental issues. Two teams have reported the transformation of apple using the phosphomannose isomerase gene as a selectable marker and mannose (1 to 2.5 g/l) as a selection pressure (Zhu et al., 2004, Degenhardt et al., 2006,). However, all the above-cited methods rely on the use of non-plant genes. A major trend in current biotechnological projects is the development of the cisgenic or intragenic approaches. These technologies aim at transforming plants with native expression cassettes only, to fine-tune the activity and/or specificity of target genes, without any introduction of non-plant DNA (Schouten and Jacobsen, 2008). In line with this objective, we decided to search for an alternative selection system for apple, based on a plant gene.

Eutypine is a toxin produced by *Eutypa lata*, the causal agent of eutypa dieback of grapevine (Amborabé et al., 2001). Eutypine is detoxified in many plant species, among which tissues of mung bean (*Vigna radiata*) exhibited the highest detoxification activity.

Purification of the eutypine reductase enzyme from mung bean led to the identification of an 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 (Guillen et al., 1998). The *Vr-ERE* protein is a monomeric NADPH-dependent oxidoreductase with a molecular mass of 36 kDa that exhibits broad substrate specificity for various natural or synthetic aldehydes (Colrat et al., 1999). The precise physiological function of this enzyme in mung bean, a non-host plant of *Eutypa lata*, is still unclear.

Transgenic rootstock grapevine 110 Richter *Vitis berlandieri* x *V. rupestris* was successfully transformed with the *Vr-ERE* gene under the control of the 35S promoter, using kanamycin as selective agent. Three clones constitutively expressing *Vr-ERE* demonstrated enhanced resistance to eutypine at the *in vitro* stage (Legrand et al., 2003).

The fact that a variety of phytotoxic compounds containing an aldehyde group can act as substrates for the *Vr-ERE* enzyme opens the possibility to select one of them in order to use *Vr-ERE* as an alternative selection system for plant transformation. As a first step of our project, we report here the effects of overexpressing the *Vr-ERE* in transgenic apple plants.

MATERIAL AND METHODS

Plant and bacterial material

The apple variety used in this study, Greensleeves, was selected from a cross between James Grieve and Golden Delicious; this variety is known as very easy to transform since the pioneer work of James et al. (1989).

The bacterial strain used in the transformation experiments was *Agrobacterium tumefaciens* strain EHA105 containing the ternary plasmid pBBR1MCS-5 with a constitutive

100 *virG* gene (van der Fits et al., 2000). The *Vr-ERE* gene from the pGA-Vr-ERE binary plasmid
101 (kindly provided by Dr. Roustan, INRA Toulouse) was placed under the control of a
102 duplicated cauliflower mosaic virus (CaMV) 35S promoter (Kay et al., 1987) and cloned in a
103 pCAMBIA2301 plasmid, to generate the binary vector pCambiaVr-ERE-GUS (Figure 1-A),
104 which was used for all the transformation experiments.

106 ***In vitro* culture, regeneration and transformation**

107 Proliferating shoot cultures were established on Murashige and Skoog (MS) (1962)
108 medium supplemented with 0.5 mg/l 6-benzyladenine (BA) and 0.1 mg/l 3-indolebutyric acid
109 (IBA). The youngest leaves of 4-week-old shoots were placed with the adaxial side down on a
110 regeneration medium consisting of MS medium containing 5 mg/l thidiazuron (TDZ), 0.5
111 mg/l IBA, solidified with 0.3% gelrite, and supplemented with varying concentrations of
112 aldehyde reductase substrates. Explants were incubated in the dark and, after one month,
113 subcultured on a similar medium, still in the dark. Callus formation, extent of necrosis, rate of
114 regeneration and number of buds per explant were assessed after two months.

115 Transformation experiments were conducted as previously described on apple (Norelli
116 et al., 1996). In brief, the youngest leaves of 4-week-old *in vitro* shoots were crushed with
117 nontraumatic forceps, and then immersed in the inoculum prepared at a concentration of 10^9
118 bacteria/ml for five minutes. Leaves were then blotted and plated on cocultivation medium for
119 three days in the dark. Leaves were then wounded transversely with a scalpel and plated
120 adaxial side down on regeneration medium containing cefotaxime at 200 mg/l, timentin at 100
121 mg/l and the appropriate selection agent (kanamycin or aldehydes). The explants were
122 transferred to fresh medium every month for six months and kept in the dark.

123 Selected transgenic clones were rooted by auxin treatment (IBA 3 mg/l for 7 days)
124 followed by transfer to hormone-free medium. Acclimatization was performed in greenhouse,

by placing the rooted plants in a peat/perlite mixture and progressively decreasing the relative humidity.

Ploidy level assessment

The ploidy level of transgenic clones and controls was estimated by flow cytometry. Nuclei were isolated from *in vitro* leaves by manual chopping with a razor blade into the buffer (Brown et al. 1991) with 2% (v/v) 4',6 diamidino-2-phenylindole (Cheminex) followed by filtration through 20 µm nylon mesh and analyzed by the cytometer (Cell analyzer II; Partec, Münster, Germany). Pea leaf nuclei were used as internal reference.

Molecular analyses of transgenic clones

The presence of the *Vr-ERE* gene was confirmed by PCR using specific oligonucleotides (F1 : forward, 5'-TTCTTCTCGAACGCGGCTAC-3' and R1 : reverse, 5'-GTCTGCGGATCTTTGGCATC-3') after DNA extraction from leaves from *in vitro* and greenhouse-grown plants using the DNeasy® Plant Mini Kit (Qiagen).

For real-time PCR analysis (Q-PCR), RNA was isolated from young leaves from greenhouse-grown plants (2 biological repeats) using the NucleoSpin® RNA Plant kit (Macherey-Nagel) according to the manufacturer's recommendations. Nucleic acids were quantified (Nanodrop Technologies Inc., Wilmington, USA) and their quality was checked by electrophoresis on agarose gel. 2 µg RNA were denatured for 10 min at 70 °C with 0.5 µg of oligo(dT)15 (Promega) and then subjected to reverse transcription with 200 U of MMLV-RT (Promega), 0.5 mM of each dNTP in a final volume of 30 µl for 1 h at 42 °C. Absence of genomic DNA in the cDNA was checked by PCR with the reverse primer R1 (as described above) and a forward primer in the sequence of the 35S promoter (F2 : 5'-CCTCGTGGGTGGGGGTCC-3'.

Real-time PCR reactions were performed in duplicate using 3 µl of retrotranscription (RT) product in a final volume of 15 µl containing 1X IQ SYBR Green Supermix (Bio-RAD), and 0.2 µM of each primer. Primers for amplification of Vr-ERE were the same as for PCR analysis (F1 and R1). Primers for the reference gene *Actin* (accession CV151413) were forward 5'-CAACCTCTCGTTCTGTGATAAT-3' and reverse 5'-GCATCCTTCTGTCCCATCC-3'. Amplifications were performed using an Opticon 4 RealTime PCR detector (Bio-RAD) as follows: 95°C for 3 min; 40 X [95 °C for 15 s; 62 °C for 1 min]. The amplification specificity was verified by a final dissociation curve ranging from 60 to 95 °C. Amplification and dissociation curves were monitored and analyzed with an Opticon Monitor (Bio-RAD). The amount of plant RNA in each sample was normalized using the *Actin* gene as reference. The relative expression level was calculated according to Pfaffl (2001), using the background signal observed in the Greensleeves control as calibrator. The standard error was calculated from four repeats per sample (2 replicates from independent RNA extraction x 2 technical repeats).

For western-blot analysis, 1.5 g of young leaves from greenhouse-grown plants was ground with 5 ml of extraction buffer (Tris-HCl 100 mM, pH 7.5, 2.5 mM dithiothreitol, 2.5% polyvinylpolypyrrolidone). After centrifugation at 13000 g for 20 min at 4°C, the supernatant was immediately used or stored at -80 °C. Protein content was determined using a bicinchonic acid dye reagent (Sigma) and bovine serum albumin as standard. Aliquots (20 µg) of protein extract from control and transgenic clones in Laemmli buffer were separated on 12% SDS-tricine polyacrylamide (w/v) gel according to the discontinuous procedure of Schägger and von Jagow (1987). After electrophoresis, proteins were blotted onto Hybond C nitrocellulose membrane (Amersham) by passive transfer. Polyclonal rabbit anti-Vr-ERE antiserum was used. Vr-ERE was detected with the enhanced chemiluminescence detection system (ECL,

Amersham) using horseradish peroxidase-labeled secondary antibody, according to the manufacturer's instructions.

Measurement of aldehyde reductase activity

Proteins were extracted as it is described for western-blot analysis. Aldehyde reductase activity was assessed spectrophotometrically by measuring the rate of enzyme-dependent decrease of NADPH absorption at 340 nm for 16 minutes, as described previously (Colrat et al., 1999). Briefly, the reaction mixture consisted of 200 mM Na₂HPO₄ / 100 mM citric acid buffer, pH6.5, 80 μM NADPH, 50 μg/ml of protein extract and substrate at a concentration of 10 times the K_M value of each substrate as defined in Colrat et al. 1999. Thirteen substrates were tested: benzaldehyde (BD), 2-hydroxy-benzaldehyde (2HBD), 3-hydroxy-benzaldehyde (3HBD), 4-hydroxy-benzaldehyde (4HBD), 3-methoxy-benzaldehyde (3MBD), 4-methoxy-benzaldehyde (4MBD), 3-nitro-benzaldehyde (3NBD), 4-nitro-benzaldehyde (4NBD), 3-fluoro-benzaldehyde (3FBD), 4-pyridoxy-benzaldehyde (4PBD), tolylaldehyde (Tolyl), decylaldehyde (Decyl), coniferylaldehyde (Conyl). A control without protein extract was used to evaluate the rate of NADPH degradation. Assays were performed in quadruplicate, on a single extract for each transgenic clone.

Statistical analyses

Statistical analyses - including ANOVA and Dunnett's test to compare the rate of bud regeneration - performed with SAS 9.1 software (SAS institute, Cary, N.C.)

RESULTS

Transformation experiments with the Vr-ERE transgene

Production of transgenic plants constitutively expressing *Vr-ERE* was attempted on the apple cultivar Greensleeves using kanamycin as selection pressure. In total, 156 buds were regenerated from a total of 410 leaf explants. Only one regenerating bud per leaf was subsequently micropropagated under the light, to insure that only independent transformation events were analyzed. Seventeen buds developed into plantlets under kanamycin selection pressure. Ploidy level determined by flow cytometry revealed only diploid clones among the regenerants. All diploid clones that survived on kanamycin were checked for the presence of the *Vr-ERE* gene by PCR analysis (Figure 1-B). Unlike the control, a specific fragment of the expected size (220 bp) was amplified from the genomic DNA extracted from all transgenic clones except one (GL6). The final transformation efficiency was 3.9% of the inoculated explants producing a diploid transgenic clone.

Eight *Vr-ERE* transgenic clones from Greensleeves were rooted and acclimatized in the greenhouse with an average survival rate of 95%. Growth of these clones was monitored for seven months (Figure 2-A). The length and morphology of the shoots in most clones were similar to those in the control. Two clones (GL1 and GL5) were slightly shorter than the control. All the subsequent analyses were performed on this sample of eight transgenic clones.

Level of expression of the Vr-ERE transgene in transgenic apple plants

Real-time PCR was used to quantify *Vr-ERE* mRNA in Greensleeves transgenic clones growing in the greenhouse. Only background signal was obtained for the non-transgenic control clone, whereas all transgenic clones revealed varying levels of expression of *Vr-ERE* (Figure 2-B). According to these results, the tested transgenic clones were separated into three groups: one clone was a low expressor (GL3), three clones were high

expressors (GL5, 7 and 11), the remaining four clones were intermediate (GL1, 10, 12 and 13).

Western blot analysis of proteins extracted from leaves of *Vr-ERE* transgenic clones from Greensleeves, using antibodies raised against the Vr-ERE protein, revealed a 36 kDa protein corresponding to the expected size of the Vr-ERE protein (Figure 3). This band was absent in the Greensleeves control (GLC), and strongly detected in a protein extract of mung bean (*Vigna radiata*). This band was clearly detected in seven transgenic clones. The band was absent or below the detection threshold in clone GL3. In addition, a non-specific band of about 26 kDa was detected in all samples.

Aldehyde reductase activity in the *Vr-ERE* transgenic apple plants

Determination of the eutypine reductase activity due to the expression of the *Vr-ERE* transgene could not be performed directly with eutypine as a substrate, as this compound was not available. However, the previous studies of Guilen et al. (1998) and Colrat et al. (1999) demonstrated that Vr-ERE has a broad range of substrates including various aromatic and aliphatic aldehydes. In the first step, aldehyde reductase activity was determined using protein extracts from leaves of greenhouse-grown plants of three apple cultivars Greensleeves, Ariane and Galaxy and five substrates of Vr-ERE (Figure 4). Enzymatic activities varied between 2 and 14 nkatal.mg⁻¹ protein, which is higher than the value reported for the grapevine Richter tested with eutypine by Legrand et al. (2003). Our results indicated marked variability between apple genotypes, Galaxy having the highest average activity, followed by Ariane, then Greensleeves. Furthermore, an interaction between genotype and substrate was detected.

In the second step, detailed aldehyde reductase enzymatic activity measurements, using 13 different substrates of Vr-ERE, were performed on eight apple *Vr-ERE* transgenic clones from Greensleeves. A summary of the results is given in Table 1. Most of the apple

transgenic clones displayed activities similar to those of the control for the majority of substrates, and only a few positive or negative differences in one to three substrates. However, the two clones (GL11 and GL12) expressed significantly more activity than the control with the large majority of substrates. However, even for these two clones, the highest aldehyde reductase activity was only about 12 nkatal.mg⁻¹ protein, which is significantly higher than the activity of the non-transgenic control Greensleeves, but within the range of variation of activity measured in the other non-transgenic apple cultivars Galaxy and Ariane (Figure 4).

Tolerance to aldehydes in the *Vr-ERE* transgenic apple plants

Tolerance to aldehydes in Greensleeves transgenic clones expressing the Vr-ERE transgene was assessed during micropropagation and during adventitious bud regeneration experiments, using several different substrates of Vr-ERE. Toxicity of several aldehyde compounds (BD, 3FBD, 3MBD, 3NBD, 4PBD) was observed on the Greensleeves control clone, at doses around 1 mM. However, none of these experiments revealed a significant increase in tolerance in all the transgenic clones tested, compared to the Greensleeves control. As an example, the results obtained with clones GL11 and GL12, the two clones that expressed significantly higher aldehyde reductase activity with a majority of substrates, are given in Tables 2 and 3. No difference in tolerance to a range of 3NBD concentrations was detected during micropropagation, and all explants died on 1.5 mM 3NBD (Table 2). Similarly, during regeneration experiments from leaves of these two clones necrosis was very similar to that induced on control leaves by concentrations of 3MBD (0.5 to 1.5 mM) and 3FBD (0.25 to 1 mM). The regeneration potential of these two clones on a medium without any selection pressure was lower than in the control, and regeneration was totally inhibited at

doses of 3MBD and 3FBD lower than the inhibitory concentrations for the control leaves (Table 3).

DISCUSSION

Because stable transformation of plant cells usually occurs at low frequencies, selection is a necessity to recover transformants of many plant species. So far, the *nptII* selectable marker conferring resistance to kanamycin has been widely used in many crops. This antibiotic is widely dispersed in nature and has very limited therapeutic use (Miki and McHugh, 2004). Consequently the rationale for the development of new selection systems is to improve a general public perception and acceptability. Among the alternative systems currently being developed are positive selection systems such as mannose selection (Wallbraun et al. 2009), and site-specific recombination systems that generate marker-free plants (Thirukkumaran et al. 2009, Khan et al. 2010). Most of the alternative systems studied on apple are based on bacterial genes (*bar*, *PMI*). Currently the presence of coding sequences of bacterial origin is a concern for the safety of transgenic plants. The *Vr-ERE* gene, from *Vigna radiata* is a natural detoxification system of plant origin. It thus appears to be an attractive alternative for apple transformation. The purpose of the present work is to study the effects of overexpression of the *Vr-ERE* gene in apple transgenic plants, and thus to evaluate the feasibility of using *Vr-ERE* as a selectable marker.

Our results have demonstrated that the production of viable transgenic apple plants expressing the *Vr-ERE* gene is possible using kanamycin selection. Transformation efficiency in this experiment was in the same range as previously reported transformation efficiencies obtained in the same laboratory, on cvs. Galaxy and Ariane (Faize et al., 2003, 2004). Our

results on acclimatized transgenic plants also indicate that there is no general deleterious effect of the overexpression of *Vr-ERE* in apple, whereas in grape, overexpression of *Vr-ERE* had a clear morphological effect, with loss of apical dominance (A. Bouquet, personal communication).

RT-PCR analyses indicated that the *Vr-ERE* transgene was expressed at variable RNA level in Greensleeves transgenic clones. Western blot analyses revealed normal translation of *Vr-ERE* mRNAs in most Greensleeves transgenic clones, but marked variability among clones regarding the amount of detectable protein. A precise correlation between mRNA and protein levels of expression could not be calculated, but the general trend of the results indicates coherence between these two evaluations of transgene expression. In particular, no *Vr-ERE* protein signal was detected in clone GL3, which also had the lowest mRNA level.

Detailed aldehyde reductase enzymatic activity measurements, using 13 different substrates of *Vr-ERE*, revealed only a very limited increase in aldehyde reductase activity conferred by the expression of the *Vr-ERE* transgene. Furthermore, micropropagation and regeneration experiments revealed no increase of tolerance to aldehydes in Greensleeves transgenic clones. This low efficiency of *Vr-ERE* expression in transgenic apple clones may have the following explanation. The precise function of *Vr-ERE* in its donor organism (*Vigna radiata*) is still unknown. A search in protein sequence databases indicated that the *Vr-ERE* protein presents significant similarities to enzymes of two secondary metabolic pathways: lignin with Cinnamyl alcohol dehydrogenase (CAD) and Cinnamoyl-CoA reductase (CCR), and flavonoid with dihydroflavonol-4-reductase (DFR) (Guillen et al. 1988). Amino acid similarity with the *Malus domestica* CCR is 71%. Because of this similarity to endogenous *Malus* sequences, a certain degree of silencing of the transgene can be expected. However, partial silencing of *Vr-ERE* in transgenic plants cannot be solely responsible for the lack of tolerance to toxic aldehyde compounds, since the presence of the *Vr-ERE* transgenic protein

was clearly demonstrated by western-blot analysis. Preliminary transformation experiments on cultivars Galaxy and Ariane using BD as a selection pressure indicated insufficient selection in favor of transgenic cells (data non shown).

Several positive selection systems based on toxic metabolites intermediates have been developed for the production of transgenic plants. However, none of these alternative selectable marker genes has yet progressed to the level of the major antibiotic or herbicide marker genes (Miki and McHugh, 2004). Our work with transgenic apple plants overexpressing the detoxifying gene *Vr-ERE* illustrates some of the difficulties in developing an alternative selection pressure. Further studies will be needed to develop an efficient selection system based on a plant gene. The following criteria can be proposed: 1) narrow substrate specificity of the detoxifying gene, 2) high toxicity of the chosen substrate in the target species, 3) limited similarity with endogenous genes of the target species, 4) limited modification of the transgenic plant endogenous metabolism, 5) absence of composition or growth abnormalities in the transgenic plants.

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References

- Amborabé BE, Fleurat-Lessard P, Bonmort J, Roustan JP, Roblin G (2001) Effects of eutypine, a toxin from *Eutypa lata*, on plant cell plasma membrane: possible subsequent implications in disease development. Plant Physiol. Biochem 39: 51-58
- Brown SC, Bergounioux C, Tellet S, Marie D (1991) Flow cytometry of nuclei for ploidy and cell cycle analysis. In: Negrutiu I and Charti-Cherti (eds) Biomethods: a laboratory guide for cellular and molecular plant biology, Birkhaeuser Verlag, Basel, pp. 326-345
- Brown SK, Maloney KE (2005) *Malus x domestica* Apple. In: Litz R.E. (ed) Biotechnology of Fruit and Nut Crops, CAB Int., New York. pp. 475-511
- Colrat S, Latché A, Guis M, Pech JC, Bouzayen M, Fallot J, Roustan JP (1999) Purification and characterization of a NADPH-dependent aldehyde reductase from mung bean that detoxifies eutypine, a toxin from *Eutypa lata*. Plant Physiol 119: 621-626
- Degenhardt J, Poppe A, Montag J, Szankowski I (2006) The use of phosphomannose-isomerase/mannose selection system to recover transgenic apple plants. Plant Cell Rep 25: 1149-1156
- Faize M, Malnoy M, Dupuis F, Chevalier M, Parisi L, Chevreau E (2003) Chitinases of *Trichoderma atroviridae* induce scab resistance and some metabolic changes in two cultivars of apple. Phytopathol 93: 1496-1504
- Faize M, Sourice S, Dupuis F, Parisi L, Gautier MF, Chevreau E (2004) Expression of wheat puroindoline-b reduces scab susceptibility in transgenic apple (*Malus x domestica* Borkh.). Plant Sci 167: 347-354
- Guillen P, Guis M, Martinez-Reina G, Colrat S, Dalmayrac S, Deswarte C, Bouzayen M, Roustan JP, Fallot J, Pech JC, Latché A (1998) A novel NADPH-dependant aldehyde

- 373 reductase gene from *Vigna radiata* confers resistance to the grapevine fungal toxin
374 eutypine. Plant J 16: 335-343
- 375 James DJ, Passey AJ, Barbara DJ, Bevan MV (1989) Genetic transformation of apple (*Malus*
376 *pumila* Mill.) using disarmed Ti-binary vector. Plant Cell Rep 7: 658-666
- 377 Khan RS, Thirukkumaran G, Nakamura I, Mii M (2010) *Rol* (root loci) gene as a positive
378 selection marker to produce marker-free *Petunia hybrida*. Plant Cell Tiss Org Cult
379 101:279-285.
- 380 Kay R, Chan A, Daly M, McPherson J (1987) Duplication of CaMV 35S promoter sequences
381 creates a strong enhancer for plant genes. Science 236: 1299-1302
- 382 Legrand V, Dalmayrac S, Latché A, Pech JC, Bouzayen M, Fallot J, Torregrossa L, Bouquet
383 A, Roustan JP (2003) Constitutive expression of *Vr-ERE* gene in transformed
384 grapevines confers resistance to eutypine, a toxin from *Eutypa lata*. Plant Sci 164:
385 809-814
- 386 Micki B, McHugh S (2004) Selectable marker genes in transgenic plants: applications,
387 alternatives and biosafety. J Biotechnol 107: 193-232
- 388 Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco
389 tissue culture. Physiol Plant 15: 473-497
- 390 Norelli J, Mills JA, Aldwinckle H (1996) Leaf wounding increases efficiency of
391 *Agrobacterium*-mediated transformation of apple. HortSci 31: 1026-1027
- 392 Ogasawara H, Ueda S, Harada T, Ishikawa R, Nijizeki M, Saito K (1994) Introduction of a
393 herbicide resistant gene into an apple rootstock with *Agrobacterium tumefaciens*. Bull
394 Fac Agric Horisaki Univ 57: 1-8
- 395 Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-
396 PCR. Nucleic Acids Res 29(9):e45 doi:10.1093/nar/29.9.e45

- Schägger H, Von Jagow G (1987) Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in range from 1 to 100 kDa. Anal. Biochem 166: 368-379
- Schouten HJ, Jacobsen E (2008) Cisgenesis and intragenesis, sisters in innovative plant breeding. Trends Plant Sci 13: 260-261
- Szankowski I, Bribiva K, Fleschhut J, Schönherr J, Jacobsen HJ, Kiesecker H (2003) Transformation of apple (*Malus domestica* Borkh.) with the stilbene synthase gene from grapevine (*Vitis vinifera* L.) and a PGIP gene from kiwi (*Actinidia deliciosa*). Plant Cell Rep 22: 141-149
- Thirukkumaran G, Khan RS, Chin DP, Nakamura I, Mii M (2009) Isopentenyl transferase gene expression offers the positive selection of marker-free transgenic plant of *Kalanchoe blossfeldiana*. Plant Cell Tiss Org Cult 97:237-242.
- Van der Fits L, Deakin EA, Hoge JH, Memelink J (2000) The ternary transformation system : constitutive *virG* on a compatible plasmid dramatically increases *Agrobacterium*-mediated plant transformation. Plant Mol Biol 43: 495-502
- Wallbraun M, Sonntag K, Eisenhauer C, Krzcal G, Wang YP (2009) Phosphomannose-isomerase (*pmi*) gene as a selectable marker for *Agrobacterium*-mediated transformation of rapeseed. Plant Cell Tiss Org Cult 99:345-351
- Zhu LH, Li XY, Ahlman A, Xue ZT, Welander M (2004) The use of mannose as a selection agent in transformation of the apple rootstock M26 via *Agrobacterium tumefaciens*. Acta Hort 663: 503-506

Table 1: Summary of enzymatic activity measurements of Vr-ERE transgenic clones tested with 13 substrates of Vr-ERE

Génotype	List of tested Vr-ERE substrates												
	BD	2HBD	3HBD	4HBD	3MBD	4MBD	3NBD	4NBD	3FBD	4PBD	Tolyl	Decyl	Conyl
GL1	n.s.	n.s.	n.s.	n.s.	+++	n.s.	+	n.s.	n.s.	n.s.	++	-	n.s.
GL3	n.s.	n.s.	n.t.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	++	n.s.	n.s.	n.s.
GL5	n.s.	n.s.	n.s.	n.s.	n.s.	---	n.s.	-	n.s.	n.s.	--	++	n.s.
GL7	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	+++	n.s.
GL10	+++	n.s.	n.s.	n.s.	n.s.	n.s.	+++	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
GL11	+++	+++	n.s.	+++	++	+++	+++	+++	+++	+++	n.s.	+++	n.s.
GL12	++	+++	++	n.s.	+++	n.s.	++	+++	n.s.	+++	+++	+++	n.s.
GL13	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.t.

GL1 to GL13: apple Vr-ERE transgenic clones from Greensleeves. Each clone was measured in quadruplicate. +, ++, +++: clones whose activity significantly higher than in the Greensleeves control, according to Dunnett's test at $p < 0.1$, 0.05 and 0.01 respectively. -, --, ---: clones whose activity was significantly lower than in the Greensleeves control, according to Dunnett's test at $p < 0.1$, 0.05 and 0.01 respectively; n.s. clones whose activity was not significantly different from that in the Greensleeves control; n.t.: non tested.

Table 2: Effect of 3-Nitro-benzaldehyde (3NBD) on micropropagation of Greensleeves Vr-ERE transgenic clones (GL11, GL12) and control (GLC)

Treatment	Dose (mM)	% shoot survival after 1 month		
		GLC	GL11	GL12
3NBD	0	100	100	100
	0.5	68	50	60
	0.75	60	45	55
	1	25	3	5
	1.5	0	0	0

Data were collected after 1 month on micropropagation medium, on 2 replicates of 20 shoots per treatment.

Table 3: Effect of 3-Methyl-benzaldehyde (3MBD) and 3-Fluoro-benzaldehyde (3FBD) on regeneration from leaves of Greensleeves Vr-ERE transgenic clones (GL11, GL12) and control (GLC)

Treatment	Dose (mM)	Necrosis score (scale from 0 to 3)			% regenerating leaves		
		GLC	GL11	GL12	GLC	GL11	GL12
3MBD	0	0	0	0	83.5	46.0	68
	0.5	0	0.37	1.12	23.0	1.5	5.0
	1	2.59	3.00	3.00	0	0	0
	1.5	3.00	3.00	3.00	0	0	0
3FBD	0	0	0	0	70.0	53.3	11.7
	0.25	0	0	0	41.7	38.3	8.3
	0.5	0	0	0	15.0	0	6.7
	0.75	1.73	2.63	0.98	5.0	0	0
	1	2.40	3.00	2.4	8.3	0	0

Data were collected after 2 months on regeneration medium, on 2 replicates of 30 leaves per treatment.

Figure legends

Figure 1: A) Schematic structure of binary vector pCambiaVr-ERE-GUS, prepared by cloning a “double35S-Vr-ERE” cassette at the *Hind*III site of pCambia2301. LB: left border; t-35S: CaMV35S terminator; *nptII*: neomycin phosphotransferase II gene; 35S: CaMV35S promoter; *Vr-ERE*: *Vigna radiata* eutypine reductase gene; *GUS*: intron-containing *uidA* gene; t-*nos*: nopaline synthase terminator; RB: right border. Primers for PCR and RT-PCR : R1 : reverse 1, F1 and F2 : forward 1 and 2. B) PCR analysis of *Vr-ERE* transgenic Greensleeves clones. Gel electrophoresis of *Vr-ERE* PCR products with the expected size of 220 bp. Ladder: 100 pb; GLC: Greensleeves control; Plasm: pCambiaVr-ERE-GUS; GL1 to 17: transgenic Greensleeves clones.

Figure 2: A) Growth of *Vr-ERE* transgenic clones from Greensleeves (GL), 7 months after greenhouse acclimatization. Clone GL6 is transgenic (presence of *nptII* transgene) but with no integration of the *Vr-ERE* transgene. Bars are the mean of 3 to 32 shoots per clone $\pm$ confidence interval at $\alpha = 5\%$. *: transgenic clones differed significantly from the GL control at $p < 0.05$ according to Dunnett's test. B) 4: Determination of transcription of *Vr-ERE* in transgenic clones of Greensleeves (GL1 to GL13) by real-time PCR. GLC: Greensleeves control. Bars are the mean of 4 repeats (2 independent RNA extractions $\times$ 2 technical repeats) $\pm$ confidence interval at $\alpha = 5\%$.

Figure 3: Western blot analysis of proteins from Greensleeves transgenic clones (GL1 to GL13). GLC: Greensleeves control, V.r.: *Vigna radiata* control.

Figure 4: Enzymatic activities measured with 5 different substrates of Vr-ERE, on control clones of Ariane, Galaxy and Greensleeves. Each bar is the mean of 5 replicates $\pm$ confidence interval at $\alpha = 5\%$.

A

B

Figure 1

Figure 2

Figure 3

Figure 4