Grapevine (*Vitis vinifera* L.) promoter analysis by biolistic-mediated transient transformation of cell suspensions

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Summary

Expression of alcohol dehydrogenase promoters from *Vitis vinifera* L. and *Arabidopsis thaliana* was investigated in homologous and heterologous cell systems after bombardment of chimeric genes. The effect of several parameters including wounding, DNA delivery and construct type on luciferase/β-glucuronidase activities was evaluated. In parallel, alcohol dehydrogenase activity was assayed in normal and gaseous nitrogen-treated cells. Compared to *A. thaliana*, results showed large differences in reporter gene activity according to the *Adh* promoter leader sequence and the cell suspension system. The *V. vinifera* cell system was found to be appropriate to investigate *Adh* promoter functioning. A novel protocol, based on transient genetic transformation of grapevine cells by biolistic is proposed to study grapevine promoter expression, either in air or in response to anaerobiosis.

K e y   w o r d s : alcohol dehydrogenase, biolistic, cell suspension, grapevine, promoter.

Introduction

Molecular genetics of *Vitis vinifera* L. is poorly advanced compared to that of model plants. Two major groups of limiting factors can be identified: the first is related to the difficulties of identification of genetic traits because of the long term interval between generations, extended juvenility period and laborious DNA and RNA manipulation procedures; the second is related to the low reliability of biotechnologies (*i.e.* stammering of protoplast or cell suspension technology, scarcity of regeneration protocols, poor efficiency of genetic transformation methods). Genetic transformation represents a useful tool to investigate molecular biology or genetics, but also to create new cultivars by transferring innovative genetic characters. Because of the potential interest for the genetic improvement of grapevine cultivars, most efforts focused on stable transformation. However, despite many attempts to improve stable transformation, reliability remains poor and some cultivars are still recalcitrant (Perl *et al*. 1998). Best transformation protocols remain costly, with low efficiency and are highly time-consuming: even if performed with competent tissues, each experiment takes at least 8-12 months before regeneration of entire plants and yields to only a few transformants. Consequently, these techniques are currently feasible only if the number of transformants does not need to be high, as for instance genetic improvement or studies using plants with up/down-regulated genes. Reports about transient transformation of *V. vinifera* are rare and only focused on embryogenic tissues and cells in order to regenerate stable-transformed plants (Hebert *et al*. 1993 ; Franks *et al*. 1998). In grapevine, undifferentiated cell suspensions are easier to establish and maintain and allow the production of homogeneous tissues. However, transient transformation experiments on this material has never been reported for grapevine. Despite the limits with respect to reliability with stable transformation, this method has been successfully applied in other plants to analyze functionally sequences involved in the regulation of gene expression (Petit *et al*. 2001). The main advantage of this approach is its promptness of response, in particular in preliminary experiments on plants difficult to transform and regenerate.

The aim of this study was to develop methodic approaches allowing transient expression experiments on cell suspension culture, specially for promoter leader sequences studies. In grapevine, as information on genome is limited, little information is available on promoters. Among *V. vinifera* genes, the alcohol dehydrogenase (*Adh*) multigene family is one of the most studied at molecular and expression levels (Tesnière and Verriès 2001). Sequence analysis showed distinct promoter organizations that could be potentially related to differences observed in isogene expression patterns during fruit development (Tesnière and Verriès 2000). This gene family was also extensively studied in other plants, such as maize (Dennis *et al*. 1987) and *Arabidopsis* (Dolferus *et al*. 1994), in particular at the promoter expression level. All these previous reports indicate that *Adh* promoters are interesting candidates to study mechanisms of gene expression control in grapevine, using *AtAdh*1 promoter as reference (Dolferus *et al*. 1994). Applied to *Adh* promoter functioning, this paper reports the use of the biolistic device on cell suspensions, as a convenient DNA delivery technique to induce transient gene expression. This method revealed important differences of gene expression according to the *Adh* promoter type or length, as well as in response to anaerobiosis.

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Material and Methods

P l a s m i d   c o n s t r u c t s : The plasmid pSLluc+dE (Petit *et al.* 2001) was used as expression vector to study several promoter leader sequences. This plasmid beared the firefly luciferase gene (Ow *et al.* 1987) under the control of the LAT59 promoter and the CaMV 35S terminator. For expression plasmid construction, the LAT59 was replaced by the promoter regions described below, using *Apa*I or *Xho*I and *Nco*I sites, the later one positioning the 3'-end of the leader sequences on the start codon of *luc* gene ORF. Two plasmid controls were used in these experiments (Fig. 1 A and B): one harboring the CaMV 35S promoter fused to luciferase (*luc*) gene (pSL35S); the other with the 35S promoter fused to β-glucuronidase (*gus*) gene (pBI221, Jefferson *et al.* 1987).

V. vinifera L. *Adh* gene promoters were obtained from total DNA or genomic library by PCR based on the known sequences of *VvAdh*1 (Sarni-Manchado *et al.* 1997) and *VvAdh*2 (GenBank Acc. No. AF271074). For *VvAdh*1, the primers were respectively, 5'-CATGgggcccTTTACTTTGC-3' and 5'-CCTGccatggTTTCTTAGATACTC-3' (added *Apa*I and *Nco*I sites in lowercase letters). PCR reaction conditions were: one cycle at 94 °C for 5 min, 50 °C for 1 min, and 72 °C for 1 min, then 31 cycles at 92 °C for 1 min, 50 °C for 1 min, and 72 °C for 1 min, followed by a 15 min final extension step at 72 °C. All reactions contained, within a 25 µl final volume, 0.4 µM of each specific primer, 200 µM of deoxynucleotides and 0.75 unit of *Taq* polymerase (Promega). This fragment (295 bp) was firstly cloned into pGem-T Easy vector (Promega) to obtain pSLVA1 (Fig. 1 C).

For *VvAdh*2, the primers were 5'-AAgaattcCTATATATG TTATGTTTCTAAAATTTAGC-3' and 5'-TTggatccTTT AGGCTATATTCCTTCTCACAC-3') including an *EcoR*I and added *Bam*HI sites. PCR conditions were carried out using a first cycle of 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 2 min and then 30 cycles at 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min with a final 15 min extension step at 72 °C. The product of amplification (1115 bp) was firstly cloned into pLITMUS 29 and excised with either *Xho*I and *Nco*I or

*Apa*I and *Nco*I sites to create pSLVA21 and pSLVA22 (306 bp) respectively (Fig. 1 D and E). The *A. thaliana Adh* gene promoter (1022 bp) was cloned from pGPADH/*Bam*HI (kindly provided by R. Dolferus; Dolferus *et al.* 1994) into pSLluc+dE using adaptators containing *Xho*I and *Nco*I sites to create pSLAA1 (Fig. 1 F). PCR products were controlled by dideoxy sequencing (Sanger *et al.* 1977) using appropriate primers of pGem-T Easy or pLITMUS 29 vectors.

C e l l   p r e p a r a t i o n   a n d   p o s t - b o m b a r d m e n t   c e l l   h a n d l i n g : Callus of *V. vinifera* L. cv. Cabernet Sauvignon was initiated from stem fragments of *in vitro*-grown plantlets on a solid induction medium composed of half strength MS (Murashige and Skoog 1962) macroelements, MS microelements, Morel's vitamins, 1 g l⁻¹ casein hydrolysate, 20 g l⁻¹ sucrose, 5 µM NOA, 1 µM BAP, pH 5.8 and 7 g l⁻¹ agar. Cell suspension cultures were established from actively growing callus on a liquid medium composed of B5 macroelements (Gamborg *et al.* 1968), MS microelements, Morel's vitamins (Morel *et al.* 1970), 250 mg l⁻¹ glutamine, 20 g l⁻¹ sucrose, 1 µM kinetin, 0.5 µM NAA, pH 6, according to Hawker *et al.* (1973). Cell suspensions were cultured on a rotary shaker (150 rpm) at 25 °C with a photoperiod of 12 h (30 µmol m⁻² s⁻¹). *V. vinifera* cell cultures were subcultured weekly by transferring 30 ml of cell suspension into 250 ml Erlenmeyer flasks, containing 70 ml fresh medium (Fig. 2 A). *A. thaliana* cell suspensions (Petit *et al.* 2001) were stabilized using the same medium as above except MS vitamins were used instead of Morel's, no glutamine, no kinetin and 10 µM 2,4-D replaced 5 µM NAA. Cells were cultured under reduced light intensity (15 µmol m⁻² s⁻¹) and subcultured every 15 d by transferring 20 ml cell suspension into 250 ml Erlenmeyer flasks containing 80 ml fresh medium (Fig. 2 B). Both *V. vinifera* and *A. thaliana* cell types showed marked differences in growth rate and shape. *V. vinifera* suspensions grow as aggregates composed of irregular and large cells compared to *A. thaliana* whose cells are smaller and regular.

For particle bombardment, cells from actively growing suspension cultures (4 d-old subculture for *V. vinifera*, 10 d-old subculture for *A. thaliana*) were spread by vacuum fil-
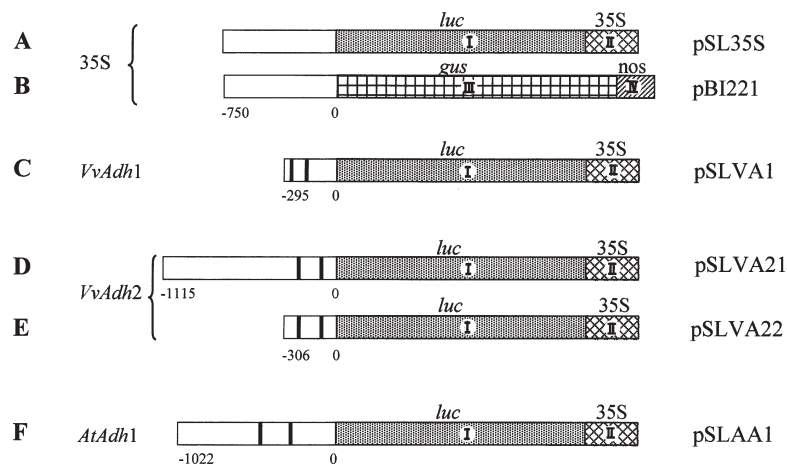

Fig. 1: Transcriptional-fusion constructs used for transient analysis of promoter leader expression levels in cell suspensions. Promoter sequences (open rectangles) from CaMV35S (**A**: pSL35S; **B**: pBIB221), *VvAdh*1 (**C**: pSLVA1), *VvAdh*2 (**D**: pSLVA21; **E**: pSLVA22) and At*Adh*1 (**F**: pSLAA1) were fused to the *luc* coding region (I) and 35S polyA region (II) except for pBIB221 fused to the *gus* coding region (III) and the nopaline synthase termination sequence (IV). Location of the anaerobic response elements (ARE) is indicated by vertical bold lines.
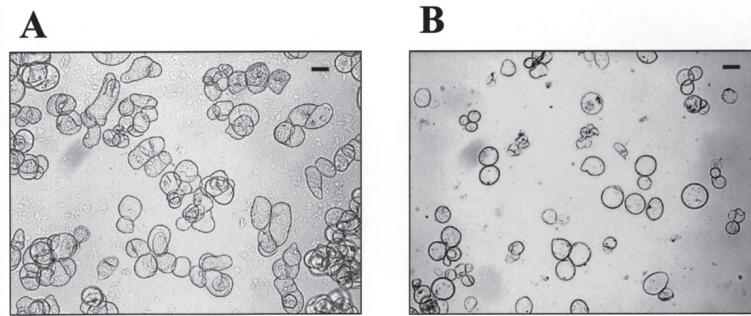
Fig. 2: Grapevine (**A**) and *A. thaliana* (**B**) cell suspensions at the exponential cell multiplication phase (bar represents 100 µm).

tration over a 45 mm diameter filter paper (Whatman n°1), pre-wet with 1 ml of culture medium. According to cell density, 1-2 ml aliquots were used to obtain homogeneous 1-2 mm high cell layers. After cell loading, filters were quickly transferred to 55 mm diameter Petri dishes, containing 10 ml solid medium (cell culture medium plus 5 g l^{-1} gelrite). Just before bombardment, filters were placed on a shock absorbing layer, composed of 5 filter papers (55 mm diameter, Prolabo) wet with a few ml of culture medium and rapidly submitted to particle delivery. Filters were then replaced on their Petri dishes and incubated under standard culture conditions for 2 h. Thereafter, cells from each filter were carefully collected to be resuspended in 4 ml liquid medium, into 10 ml volume wells (microplates Sigma). For anaerobic treatment, liquid media were flushed before use with sterile gazeous N$_2$ until saturation (2 h minimum). Immediately after transfer of cells to microplates, they were placed in a special sealed vessel maintained under continuous N$_2$ flow (approx. 1 l min^{-1}). Under these conditions, N$_2$ headspace was kept higher than 95 %. All cell suspensions in microplates were cultured for 24 h on a rotary shaker (120 rpm) at 25 °C under a photoperiod of 12 h (15 or 30 µmol m^{-2} s^{-1}).

Particle bombardment: DNA coating of microcarrier and He biolistic particle-delivery system were performed as described by SAVINO *et al*. (1997), with a few modifications. Gold microcarriers (1 µm, Bio-Rad) were washed in 100 % ethanol and resuspended in 50 % glycerol at 130 µg µl^{-1}. For their DNA coating, 6.25 µg DNA of each promoter-*luc* construct and 6.25 µg of the internal standard plasmid (pBI221) were added to 25.5 µl of particle suspension, 32 µl of CaCl$_2$ (2.5 M) and 12.5 µl of spermidine (0.1 M), mixed and then incubated on ice for 5 min. The mix was briefly centrifuged and 64 µl of supernatant were discarded. The remaining mixture (18.5 µl) was gently homogenised and maintained on ice until use. For each bombardment, 2 µl of DNA-carrier mix were placed in the center of a stainless steel filter holder (200 µm holes, Millipore) and propelled under a 2.5 kPa vacuum at 400 kPa of He into cells positioned 21 cm below using a home-made particle delivery apparatus. For each condition studied (cell origin, construct x treatment), 6 bombardments were independently performed, 5 with DNA and one without DNA as background control. Each experiment was repeated at least twice using different cell suspension cultures and analyzed separately.

Protein determination and ADH, LUC and GUS assays: For protein content and enzyme activity determinations, the 4 ml cell suspensions were pelleted by centrifugation (2500 *g*, 5 min at 10 °C) and washed with 5 ml 0.1 M phosphate buffer, pH 7.8. After a second centrifugation, cells were resuspended in 400 µl extraction buffer (0.1 M sodium phosphate, pH 7.8, 1 mM DTT and 0.1 % (v/v) Triton X-100). Following 2 ∞ 5 s sonication, lysates were centrifuged (13,000 *g*, 10 min at 4 °C) and 500 µl of supernatant were recovered. Protein contents were determined using Bradford's dye method (BRADFORD 1976) on 5 µl extract, with BSA as standard. ADH activity assays (µmol min^{-1} µg^{-1} prot) were performed according to MOLINA *et al*. (1987), by measuring the reduction rate of acetaldehyde (5 mM) at 340 nm. The assay mixture contained 5-10 µl extract and 50 mM sodium phosphate buffer (pH 5.8), 0.24 mM NADH. LUC and GUS activities were determined on 20 µl extract each, with a luminometer (1203 Bio-Orbit, Turku, Finland), using the GUS light kit (Tropix) and the luciferase assay system (Promega) according to the manufacturer's instructions. Activities were obtained in arbitrary light units; unless mentioned, results corresponded to ratio averages between LUC and GUS activities.

Results and Discussion

Evaluation of biolistic on cell suspensions: A preliminary verification of the effect of biolistic processes on the ADH and reporter gene activities in *V. vinifera* and *A. thaliana* cell suspensions was carried out (Fig. 3). Bombardments were performed using microparticles without DNA or coated with either 35S (pSL35S) or *VvAdh*2 (pSLVA21) or *AtAdh*1 (pSLAA1) promoter constructs. To overcome data dispersion related to the biolistic method, these constructs were co-bombarded with the control plasmid containing CaMV 35S promoter fused to *gus*. In these conditions, *V. vinifera* cells exhibited a constitutive ADH activity (Fig. 3 A). Neither wounding resulting from bombardment, nor DNA introduction, nor even construct type have a significant effect on this activity. In all cases, ADH was markedly enhanced if DNA-transformed cells were placed under anaerobiosis (N$_2$). In a previous study focused on the effect of hypoxia in grapevine, berry cell cultures (cv. Gamay Fréaux) showed a similar response to limited oxygen supply (TESNIÈRE *et al*. 1993). In parallel, *A. thaliana* cells also exhibited a constitutive ADH activity, but always at a lower level than those of *V. vinifera*
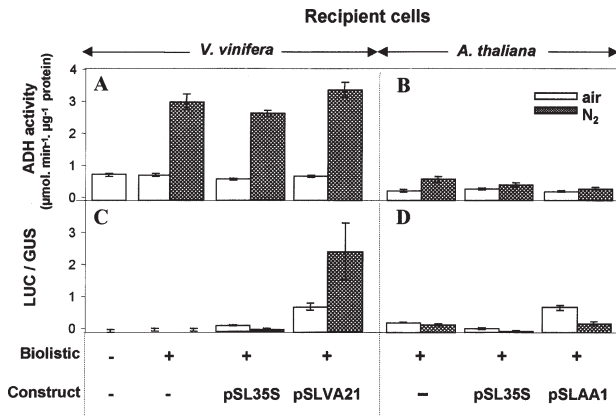
Fig. 3: Effect of biolistic and construct types on ADH activity (**A** and **B**) and transient expression levels of chimeric genes (**C** and **D**) into *V. vinifera* or *A. thaliana* cells submitted to N_2 for 24 h. The constructs were co-bombarded with the control plasmid pBI221. The LUC/GUS ratios corresponded to normalized mean values and SE bars of 5 independent bombardments.

(Fig. 3 B). Moreover, a slight response to anaerobiosis was observed. The low ability of *A. thaliana* cells to exhibit a stress response when placed under anaerobiosis has previously been reported by McKendree and Ferl (1992).

For promoter activity analysis, *luc* was chosen as reporter gene because its activity is known to be highly sensitive and substrate-specific (Gould and Subramani 1988). In both cell types, background LUC activity was low and unchanged after bombardment (Fig. 3 C and D). This indicates no intrinsic LUC-like activity. However, the background level slightly varied between and within species. When *V. vinifera* and *A. thaliana* cells were transformed with 35S-*luc* construct, they expressed similar reporter gene activity (expressed as LUC/GUS). Although the 35S promoter is known to have a stable and constitutive expression (Odell *et al*. 1985), LUC/GUS unexpectedly quenched with anaerobic treatment in both types of cell suspensions. Since 35S GUS activity should be constant per µg protein and to avoid variability between experiments, LUC/GUS data were normalized using a weighing factor, considering each ratio of 35S GUS values per µg protein within experiment as equivalent to 1. Similar normalization has already been used in other studies, as for instance in sugarbeet cells (Ingersoll *et al*. 1996) and tomato tissues (Baum *et al*. 1997).

Expression of ADH promoters from *V. vinifera* and *A. thaliana* was evaluated in homologous cell systems and compared to 35S (Fig. 3 C and D). In air, *VvAdh*2 and *AtAdh*1 promoters, pSLVA21 and pSLAA1, respectively, exhibited similar constitutive activities, with 4 to 7-fold higher expression than pSL35S. Both promoters responded to anaerobiosis but with different patterns: whereas the *VvAdh*2 promoter exhibited an expression 3 times higher than in air (Fig. 3 C), that of *AtAdh*1 decreased (Fig. 3 D). In *V. vinifera* cells, promoter expression response varied in the same way as ADH activity, suggesting that *Adh* expression mainly resulted from a positive transcriptional control, whereas in *A. thaliana* cells this relationship remained unclear.

A d h p r o m o t e r l e a d e r c o m p a r i s o n : In air, the *Adh* leader version did not modify ADH activity of grape-

vine cells (Fig. 4 A). Compared to pSLVA21, the pSLVA22 construct, which contained a shorter version of the *VvAdh*2 promoter, showed a reduced LUC/GUS expression level but conserved a similar response to anaerobiosis (Fig. 4 B). On the contrary, the same length segment of *VvAdh*1 (pSLVA1), showed a very low promoter activity level and no response to anaerobiosis. With pSLAA1, driven by the promoter of *A. thaliana Adh*1 of similar length as pSLVA21, the reporter gene expression in air and under N_2, was intermediary between both versions of *VvAdh*2. These results proved that *Adh* promoter activities are regulated differently. In air, the highest constitutive expression was observed with the larger *VvAdh*2 fragment. Under anaerobiosis, all tested *Adh* leader sequences lead to enhanced activities of the same extent, except that of *VvAdh*1. In *V. vinifera* cell suspensions, while ADH and *VvAdh*2 promoter activities concomitantly increased, those of *VvAdh*1 remained unlinked. Activity of a longer *VvAdh*1 leader version should be further investigated to confirm that this isogene is not mainly involved in the ADH activity of *V. vinifera* cells. However, northern blots using specific probes showed that changes in *Adh* gene expression followed *VvAdh*2 transcription (data not shown). Involvement of gene transcription in ADH activity changes has been reported earlier for grape berry cells in air and under N_2 (Tesnière *et al*. 1993).
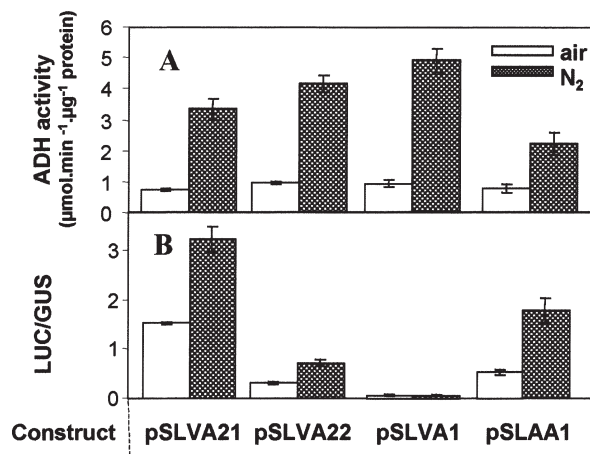

Fig. 4: Effect of *Adh* promoter types on ADH activity (**A**) and transient expression levels of chimeric genes (**B**) into *V. vinifera* cells submitted to N_2 for 24 h. For details see Fig.3.

For the *VvAdh*2 promoter, the differences of expression patterns between both versions suggested that the sequence between –1000 and –300 contained an important region for enhancement of gene transcription. However, the deletion upstream 0.3 kb had no effect on the amplitude of the anaerobiosis response, showing that the *cis*-acting regulatory signals necessary for anaerobic control of expression still reside in the –300 to 0 segment. Sequence comparisons between this region and those of plant *Adh* genes showed consensus boxes putatively involved in gene transcription control. In particular, anaerobic responsive elements (ARE) have been reported in the proximal promoter regions of *Adh* genes (Walker *et al*. 1987; Dolferus *et al*. 1994). ARE consensus sequences have been found to be highly conserved

among *Adh* promoters and more generally in promoters transcriptionally activated by anaerobiosis (Dennis *et al.* 1987). In fact, both, *VvAdh*1 and *VvAdh*2 contained two ARE consensus sequences (Fig. 1) within the 300 bp proximal region of the promoters, but with different location (Tesnière and Verriès 2001). Response of *VvAdh*2 constructs to anaerobiosis confirmed the functionality of ARE in *V. vinifera* cell system. However, the null response of the *VvAdh*1 proximal region to anaerobiosis, indicated that ARE were necessary but not sufficient to confer anaerobiosis response ability. Sequence organization variability could also be involved in the differences of response to anaerobiosis of *VvAdh* promoter isogenes in *V. vinifera* cells. Moreover, the origin and the type of cells influenced the modulation of expression. For instance, in *V. vinifera* cells *AtAdh*1 responded to anaerobiosis. In *A. thaliana* cells neither *AtAdh*1 (Fig. 3 B) nor *VvAdh* (data not shown) were found to be effective. Thus, *V. vinifera* cells produced transcription factors in response to anaerobiosis that could interact with homologous or heterologous *Adh* promoter regions, whereas these elements were lacking in *A. thaliana* cells. Whether this was due to interaction between transcription factors and ARE or other *cis*-elements remains to be determined.

This is the first report on a transient expression procedure applied to *V. vinifera* cell suspensions. The method was successfully applied to investigate *VvAdh*s and *AtAdh*1 promoter functioning. Transient transformation of *V. vinifera* cells was performed with chimeric constructs consisting of *Adh* promoters fused to the *luc* gene. The normalized LUC/GUS appeared to be a valuable system to consider promoter activity. Knowledge of the grapevine *Adh* promoter was improved: *i.e.* evaluation of promoter strength, putative regions involved in response to anaerobiosis, incidence of cell type. These preliminary results allow several hypotheses on factors that could govern *Adh* gene expression. The study will be further developed to characterize *cis* and *trans* regulatory elements and to identify putative transcription factors in response to anaerobiosis or other abiotic stress. The method could also be of advantage for application in grapevine to other promoter studies, particularly because cell suspension handling is useful to analyze the expression of genes involved in metabolism, as well as stress- and pathogen-responding genes. Moreover, this system could represent a convenient tool for preliminary evaluation of promoter constructs before considering their use in stable transformation of grapevine.

References

Baum, K.; Groning, B.; Meier, I.; 1997: Improved ballistic transient transformation conditions for tomato fruit allow identification of organ-specific contributions of I-box and G-box to the *RBCS2* promoter activity. Plant J. **12**, 463-469.

Bradford, M. M.; 1976: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye-binding. Anal. Biochem. **72**, 248-254.

Dennis, E. S.; Walker, J. C.; Llewellyn, D. S.; Ellis, J. G.; Singh, K.; Tokuhisa, J. G.; Wolstenholme, D. R.; Peacock, W. J.; 1987: The response to anaerobic stress: Transcriptional regulation of genes for anaerobically induced proteins. In: D. Von Wettstein, N. H. Chua (Eds): Plant Molecular Biology 140, 407-414. NATO ASI Series, Plenum Press, New York.

Dolferus, R.; Jacobs, M.; Peacock, W. J.; Dennis, E. S.; 1994: Differential interactions of promoter elements in stress responses of the *A. thaliana Adh* gene. Plant Physiol. **105**, 1075-1087.

Franks, T.; He, D. G.; Thomas, M.; 1998: Regeneration of transgenic *Vitis vinifera* L. Sultana plants: Genotypic and phenotypic analysis. Mol. Breed. **4**, 321-333.

Gamborg, O. L.; Miller, R. A.; Ojima, K.; 1968: Nutrient requirements of suspension cultures of soybean root cells. Exp. Cell Res. **50**, 151-158.

Gould, S. J.; Subramani, S.; 1988: Firefly luciferase as a tool in molecular and cell biology. Anal. Biochem. **175**, 5-13.

Hawker, J. S.; Downton, W. J. S.; Wiskich, D.; Mullins, M. G.; 1973: Callus and cell culture from grape berries. Hort. Sci. **8**, 388-399.

Hebert, D.; Kikkert, J. R.; Smith, F. D.; Reisch, B. I.; 1993: Optimization of biolistic transformation of embryogenic grape cell suspensions. Plant Cell Rep. **12**, 585-589.

Ingersoll, J. C.; Heutte, T. M.; Owens, L. D.; 1996: Effect of promoter-leader sequences on transient expression of reporter gene chimeras biolistically transferred into sugarbeet (*Beta vulgaris*) suspension cells. Plant Cell Rep. **15**, 836-840.

Jefferson, R. A.; Kavanagh, A.; Bevan, M. W.; 1987: GUS fusions: β-glucuronidase as a sensitive and versatile fusion marker in higher plants. EMBO (Eur. Mol. Biol. Organ.) J. **6**, 3901-3907.

McKendree, W. L.; Ferl, R. J.; 1992: Functional elements of the *Arabidopsis Adh* promoter include the G-box. Plant Mol. Biol. **19**, 859-862.

Molina, I.; Salles, C.; Nicolas, M.; Crouzet, J.; 1987: Grape alcohol dehydrogenase. II. Kinetic studies: Mechanism, substrate, and coenzyme specificity. Am. J. Enol. Vitic. **38**, 60-64.

Morel, G.; 1970: Le problème de la transformation tumorale chez les végétaux. Physiol.Vég. **8**, 189-191.

Murashige, T.; Skoog, F.; 1962: A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. **15**, 473-497.

Odell, J. T.; Nagy, F.; Chua, N. H.; 1985: Identification of DNA sequences required for activity of the cauliflower mosaic virus 35S promoter. Nature **313**, 810-812.

Ow, D. W.; Jacobs, J. D.; Howell, S. H.; 1987: Functional regions of the cauliflower mosaic virus 35S RNA promoter determined by use of the firefly luciferase gene as a reporter of promoter activity. Proc. Natl. Acad. Sci. USA **84**, 4870-4874.

Perl, A.; Eshdat, Y.; 1998: DNA transfer and gene expression in transgenic grapes. In: M. P. Tomb (Ed.): Biotechnology General Eng. Rev. **15**, 365-386. Intercept, UK.

Petit, J. M.; van Wuytsvinkel, O.; Briat, J. F.; Lobréaux, S.; 2001: Characterization of an iron dependent regulatory sequence (IDRS) involved in the transcriptional control of AtFer1 and ZmFer1 plant ferritin genes by iron. J. Biol. Chem. **276**, 5584-5590.

Sanger, F.; Nicklen, S.; Coulson, A. R.; 1977: DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**, 5463-5467.

Sarni-Manchado, P.; Verriès, C.; Tesnière, C.; 1997: Molecular characterization and structural analysis of one alcohol dehydrogenase gene (GV-*Adh*1) expressed during ripening of grapevine (*Vitis vinifera* L.) berry. Plant Sci. **125**, 177-187.

Savino, G.; Briat, J. F.; Lobréaux, S.; 1997: Inhibition of the iron-induced ZmFer1 maize ferritin gene expression by antioxidants and serine/threonine phosphatase inhibitors. J. Biol. Chem. **26**, 3319-3326.

Tesnière, C.; Romieu, C.; Vayda, M. E.; 1993: Changes in the gene expression of grapes in response to hypoxia. Am. J. Enol. Vitic. **44**, 445-451.

- -; Verriès, C.; 2000: Molecular cloning and expression of cDNAs encoding alcohol dehydrogenases from *Vitis vinifera* L. during berry development. Plant Sci. **157**, 77-88.

- -; - -; 2001: Alcohol dehydrogenase: A molecular marker in grapevine. In: K. A. Roubelakis-Angelakis (Ed.): Molecular Biology and Biotechnology of Grapevine, 131-151. Kluwer Academic Publishers, Amsterdam, The Netherlands.

Walker, J. C.; Howard, E. A.; Dennis, E. S.; Peacock, W. J.; 1987: DNA sequences required for anaerobic expression of the maize alcohol dehydrogenase 1 gene. Proc. Natl. Acad. Sci. USA **84**, 6624-6628.