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Effect of different 3' flanking regions on the activity of the *Vitis vinifera* alcohol dehydrogenase 2 promoter

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Summary

3'untranslated regions (3' UTR) are isogene specific regions which contain sequences likely playing an important role in gene expression. To evaluate the importance of these regions on *Vitis vinifera* alcohol dehydrogenase 2 (VvAdh2) expression, we designed expression cassettes containing the luciferase reporter gene under the VvAdh2 or CaMV 35S promoters and flanked by different 3' UTRs. Luciferase activity monitoring was performed through transient expression experiments, using biolistic on Cabernet Sauvignon suspension cells. Results showed that absence of the 3' region had a strong down-regulating effect on the VvAdh2 promoter activity (but not on the CaMV 35S promoter activity). The nature of the flanking 3' UTR was shown to influence expression cassette activity. Whatever the promoter, VvAdh1 and VvAdh2 terminators had similar effect on expression of luciferase in air leading to an activity level very close to that of CaMV 35S terminator. Under anaerobiosis, luciferase expression was strongly increased with all terminators, VvAdh6 3'-end inducing the highest level of expression. Functional constructs with VvAdh2 promoter and VvAdh terminators designed in this study could be used wherever grapevine-homologous, stress-stimulated cassettes should be of interest.

Key words: alcohol dehydrogenase, anaerobiosis, 3'-end, grapevine, promoter activity.

Introduction

In most plants, the alcohol dehydrogenase gene family generally consists of two to three members (DENNIS *et al.* 1985; XIE and WU 1989; LONGHURST *et al.* 1994). In grapevine, it consists of at least 4 members (TESNIERE and VERRIES, 2001). Three of them are expressed in berries, VvAdh1 being mostly expressed during the herbaceous phase of berry development (stage I), whereas VvAdh2 (TESNIERE and VERRIES 2000) and VvAdh6 (OR *et al.* 2000) are mainly expressed during the ripening phase; in particular, VvAdh2 showed a transcript level 6 - 10-fold higher in ripe berries than at the onset of ripening (TESNIERE and VERRIES 2000).

The physiological role of Adh is to limit acetaldehyde concentration and to restore NAD when respiration capaci-

ties become limited with regard to the energy demand. It could also contribute to the cytoplasmic pH homeostasis (Roberts *et al.* 1984).

To investigate which factors regulate the expression of the VvAdh genes, different experiments have been performed including promoter analyses on suspension cells transiently expressing reporter genes under either VvAdh1 or VvAdh2 control (TORREGROSA *et al.* 2002; VERRIES *et al.* 2004). In Cabernet Sauvignon suspension cells, the functioning of these two promoters was found to be different: VvAdh1 had low constitutive activity and was unresponsive to anaerobiosis whereas a significant constitutive promoter activity in air, strongly increasing under anaerobiosis, was observed for VvAdh2 (TORREGROSA *et al.* 2002). Some motifs related to the anaerobic response of the VvAdhs promoter have been described (VERRIES *et al.* 2004). The incidence of the ethylene signalling pathway on VvAdh2 transcripts level regulation was also investigated (TESNIERE *et al.* 2004), leading to a better understanding of the involvement of 5' UTRs regions in the regulation of Adh genes in grapevine.

However, if the promoter sequence is a critical element for the regulation of gene expression, *in vitro* and *in vivo* experiments (INGELBRECHT *et al.* 1989; MOGEN *et al.* 1990; MONDE *et al.* 2000; ROTHNIE 1996) proved that 3' UTRs participate in gene regulation, in particular through 3'-end synthesising and stabilisation. Using *Vitis vinifera* suspension cells transiently expressing the luciferase reporter gene fused to different terminator sequences, the effect of the 3' UTR on VvAdh2 promoter activity was investigated.

Material and Methods

Obtaining VvAdh sequences: Screening of a grapevine genomic DNA library led to the isolation of the VvAdh2 gene and its promoter region (VERRIES *et al.* 2004). The sequences of the 3'-ends of the VvAdh1 and VvAdh2 genes were obtained as described in SARNI-MANCHADO *et al.* (1997) and in TESNIERE AND VERRIES (2001), and that of VvAdh6 as described in OR *et al.* (2000).

3' terminator constructs: To determine whether the nature of the 3'-flanking region was of importance to VvAdh2 promoter activity, different constructs were designed. Terminator sequences were obtained by PCR from the 3' UTR

of the VvAdh1 and VvAdh2 genes (respectively GenBank accession numbers U36586 and AF271074) and from the VvAdh6 cDNA (GenBank accession number AF195866). Primers used to clone these sequences included *Sma*I and *Spe*I sites: F-ATCCCGGGAGATGAGAGAGTCTAA/R-AAACTAGTATCACCGAAATATTGC for 334 bp of VvAdh1 3' UTR, F-ACCCGGGAGAGATTTGCCTATTC/R-TTACTAGTCATTGGCTATGATTG for 310 bp of VvAdh2 3' UTR and F-ACCCGGGAGAGATTTGCCTATTC/R-TAAACTAGTATCCATATA for 131 bp of VvAdh6 3' UTR. All constructs with VvAdh2 as promoter were derived from the p1.1VvAdh2/35S plasmid described in VERRIES *et al.* (2004), consisting in 1.1 kb of the VvAdh2 promoter fused to the luciferase gene flanked with the CaMV 35S terminator from the reporter plasmid pSLluc+dE (PETIT *et al.* 2001). To obtain the different constructs, the CaMV 35S terminator of p1.1VvAdh2/35S was either deleted (p1.1VvAdh2/0) or replaced through *Sma*I-*Spe*I digestion/ligation by VvAdh1, VvAdh2 and VvAdh6 3' UTR, resulting in respectively p1.1VvAdh2/1, p1.1VvAdh2/2 and p1.1VvAdh2/6 constructs. All constructs with CaMV 35S as promoter were derived from the native pSLluc+dE plasmid (pCaMV35/35) with the terminator deleted (pCaMV35/0) or replaced through *Sma*I-*Spe*I digestion/ligation by VvAdh1 or VvAdh2 3' UTR (respectively pCaMV35/1 and pCaMV35/2).

Cell treatments and particle bombardment: 4-day-old suspension cells were bombarded as previously described (TORREGROSA *et al.* 2002), DNAs from reporter and reference plasmids (1:1 ratio, 6.25 µg each) being coated onto gold particles (1 µm Biorad®). Bombarded cells were further incubated at 25 °C for 24 h either in normal culture conditions or under nitrogen-saturated atmosphere. Experiments were repeated independently at least twice.

Measurement of enzyme activities and determination of proteins: Bombarded cells were harvested and homogenized in extraction buffer (0.1 M phosphate, pH 7.8, 1 mM DTT and 0.1 % Triton X-100), then sonicated (TORREGROSA *et al.*

2002). The supernatant was recovered after extract centrifugation (13,000g, 5 min, 4 °C). Luciferase and GUS activities were respectively determined using the Luciferase Assay System (Promega) and the GUS light kit (Tropix) in a 1203 Bio-Orbit (Turku, Finland) luminometer. The luciferase activity was corrected for shot-to-shot variation by delivering the luciferase constructs together with a *iudA* gene under the control of a constitutive promoter (the GUS construct) from the Arabidopsis EF1- α gene (AXELOS *et al.* 1989). Supernatant protein content and ADH activity were respectively determined as in BRADFORD (1976) with BSA as standard, and as in MOLINA *et al.* (1987) with acetaldehyde as substrate.

Results

3' UTR organization: The organization of the constructs described in the Material and Methods section was controlled by digestion and sequencing. Sequence analysis of VvAdh1 and VvAdh2 3'-ends showed that the regions between the terminal codon and the cleavage site consisted in approximately 180-190 bp (Fig. 1). It included upstream elements corresponding to the terminator transcription factor consensus (respectively UUGUUA and UGUGUA for VvAdh1 and VvAdh2), as well as the canonical polyadenylation element corresponding to the AAUAAA sequence. In both cases, the cleavage site motif was surrounded by two U-rich regions.

VvAdh6 3' UTR sequence was found almost identical to that of VvAdh2 upstream polyadenylation element site, where it was interrupted (as expected from the cDNA origin of the template).

Expression of VvAdh2 promoter in suspension cells under air and nitrogen with various 3' ends: we investigated whether VvAdh2 promoter activity was under the control of the 3'-end sequences. Constructs with VvAdh1, VvAdh2

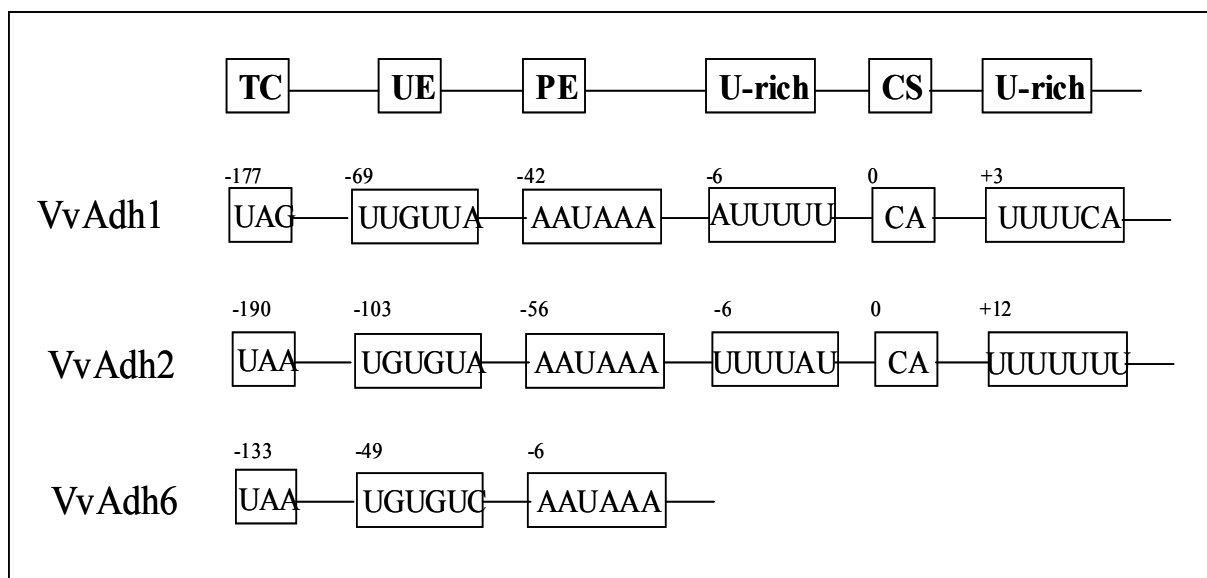


Fig. 1: Organization of VvAdh 3'-ends regions as compared to the GRABER *et al.* (1999) model of 3'-end processing signal. TC: terminal codon, UE: upstream element, PE: polyadenylation element, U-rich: uridine-rich region, CS: cleavage site.

and VvAdh6 3' UTR regions were designed and luciferase gene activity driven by the VvAdh2 promoter and CaMV 35S was determined (Fig. 2). Constructs containing either the 3' CaMV 35S terminator, or no terminator were used as controls.

The luciferase relative activity in air corresponded to the constitutive activity of the VvAdh2 promoter, which was always found to be lower than CaMV 35S-driven luciferase activity, even though different terminators were used (Fig. 2 A). With VvAdh2 as promoter, the absence of the 3' region had a tremendous down-regulating effect on luciferase expression, with a 5-30-fold decrease in reporter gene activity. Interestingly, this down-regulating effect was not observed when luciferase gene was driven by the CaMV 35S promoter (Fig. 2 A), hinting to a specific terminator action on the promoter.

The activity of p1.1VvAdh2-derived constructs was compared in air and under nitrogen (Fig. 2 B). Whatever the 3' region used, a similar effect on luciferase expression was observed in all experiments, *i.e.* a significant constitutive activity in air. Under nitrogen, a 6-fold increase in VvAdh2 promoter activity was observed while expression level ratios between the various terminators were not affected. Though very similar to VvAdh2 terminator (at least in its upstream sequence), VvAdh6 terminator led to the highest promoter activities both in air and under anaerobiosis obtained in this experiment.

Transient transformation did not modify significantly cell metabolism as indicated by suspension cells displaying similar ADH activities after bombardment with different constructs (data not shown).

In conclusion, the presence of a terminator sequence appeared essential to the functioning of the VvAdh2 promoter activity either on air (constitutive activity) or under inducing conditions. VvAdh1, VvAdh2 and CaMV 35S terminators induced the same level of luciferase relative activity. In addition, anaerobiosis generated a 5- 6-fold increase in the reporter activity (under the control of the VvAdh2 promoter) as compared with air but no interaction between anaerobiosis and terminator(s) could be observed.

Discussion

In eukaryotic cells, the 3' UTR consists in both processing signals necessary for biogenesis of functional mRNAs, and regulatory sequences that possibly affect mRNA stability (HIGGS *et al.* 1999). Recently, GRABER *et al.* (1999) investigated 3'-end control sequences *in silico*. The authors presented a broadened concept of the 3'-signals for plants, which generally was in agreement with published experimental findings. They suggested that variation in the 3'-end organization allowed modulation of gene expression. The graphical representation of the organization of the signals we found in the 3' UTRs of VvAdhs showed that VvAdh1 and VvAdh2 genes fit well to this model: presence of both the highly conserved AAUAAA polyadenylation signal (essential for both cleavage and polyadenylation according to SHEETS *et al.* 1990) and a downstream element, less conserved but generally U-rich (HART *et al.* 1985; SANFAÇON *et al.* 1991; ROTHNIE *et al.* 1994). As the complete VvAdh6 gene sequence was not available, pertinence of the above model for VvAdh6 terminator is yet to be determined.

Results indicated that presence of 3'-ends was necessary to VvAdh2 promoter activity (as inferred from luciferase reporter gene activity), while its origin (from grapevine - VvAdh1, VvAdh2 and VvAdh6- or cauliflower mosaic virus -CaMV 35S) only modulated the level of expression. However, this was apparently specific to the VvAdh2 promoter as CaMV 35S promoter did allow luciferase activity in the absence of a terminator.

The 3' UTR from VvAdh1 and VvAdh2 were very similar at the organization level whereas they were distinct at the nucleotide level (TESNIERE and VERRIES 2001). Nevertheless, under the conditions of the study, no important difference was observed between both ends in terms of promoter reporter activity level. In contrast, the higher activity observed with the VvAdh6 end suggested that additional *cis*-element(s) could be involved in the activity of the reporter gene. The fact that the nature of the 3' UTR influenced luciferase activity could reflect some interactions between the promoter and the 3'-end, leading to an increase in tran-

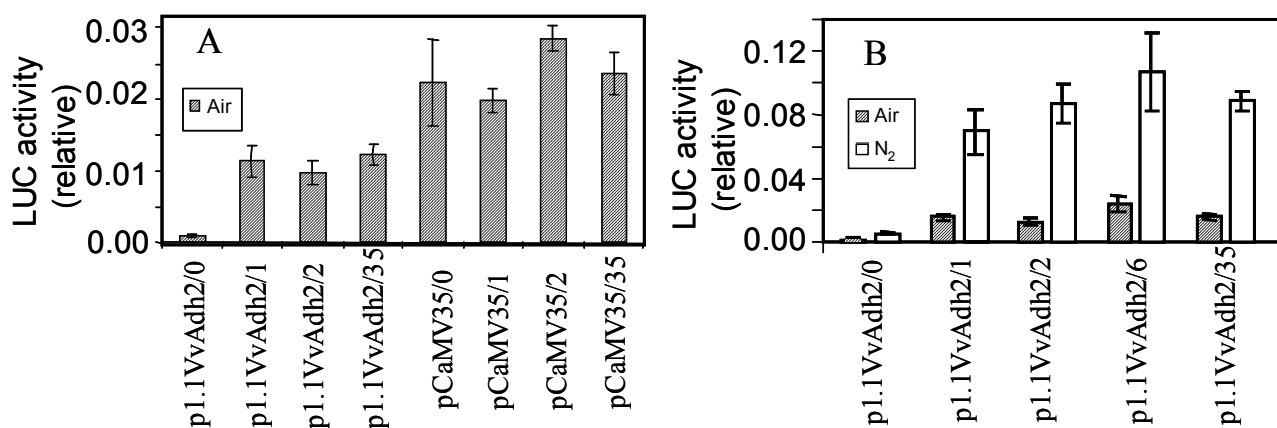


Fig. 2: Effect of different 3'-ends under VvAdh2 or CaMV 35S promoters evaluated as relative luciferase activity after transient transformation. Activities were measured in Cabernet Sauvignon suspension cells kept for 24 h in air (A) or in air and under nitrogen (B). Data are mean values of 6 bombardments within two independent experiments \pm SE.

script stability or in translation process efficiency. For instance, the 3' processing and the translation are linked as in fact translation can be regulated by interactions between the 5' and 3' UTRs and in particular by the polyadenylation status of the 3' UTR (JACOBSON and PELTZ 1996; GRAY and WICKENS 1998).

Finally, associating the VvAdh2 promoter with each of the three grapevine 3' UTR VvAdh1, VvAdh2 or VvAdh6 resulted in functional expression cassettes, indicating that these chimeric constructs could be useful tools to further study the Adh gene response to stresses and/or developmentally regulated processes. Moreover, some of the grapevine 5' and 3' UTR sequences studied here showed good efficiency when compared to CaMV 35S-based constructs (considered highly efficient), indicating that they could be used alternatively to bacterial or virus sequences if grapevine-homologous expression cassettes were required.

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