Dissection of the transcriptional regulation of grape ASR and response to glucose and abscisic acid

Amélie Saumonneau, Maryse Laloi, Magali Lallemand, Amélie Rabot and Rossitza Atanassova*

University of Poitiers, UMR CNRS 6503 LACCO, Physiologie Moléculaire du Transport des Sucres chez les Plantes, Bâtiment Botanique B31, 3 rue Jacques Fort, 86022 Poitiers, France
* To whom correspondence should be addressed. E-mail: rossitza.atanassova@univ-poitiers.fr

Received 4 August 2011; Revised 2 November 2011; Accepted 7 November 2011

Abstract

Despite the fact that the precise physiological function of ASRs [abscisic acid (ABA), stress, ripening] remains unknown, they have been suggested to play a dual role in the plant response to environmental cues, as highly hydrophilic proteins for direct protection, as well as transcription factors involved in the regulation of gene expression. To investigate further the biological positioning of grape ASR in the hormonal and metabolic signal network, three promoters corresponding to its cDNA were isolated and submitted to a detailed in silico and functional analysis. The results obtained provided evidence for the allelic polymorphism of the grape ASR gene, the organ-preferential expression conferred on the GUS reporter gene, and the specific phloem tissue localization revealed by in situ hybridization. The study of glucose and ABA signalling in its transcriptional control, by transfection of grape protoplasts using the dual luciferase system, revealed the complexity of ASR gene expression regulation. A model was proposed allowing a discussion of the place of ASR in the fine tuning of hormonal and metabolic signalling involved in the integration of environmental cues by the plant organism.

Key words: Allelic polymorphism, ASR transcriptional regulation, dual luciferase reporter system, glucose and ABA signalling, grapevine, promoter functional analysis, phloem conducting complex.

Introduction

Primary stresses, drought, salinity, cold, and heat, are usually interconnected and they affect two groups of stress-regulated proteins. The first group encompasses proteins whose activity enables the plant to survive, such as proteins required for osmotic and ion homeostasis, and scavenging of activated oxygen species, and highly hydrophilic proteins for direct protection. The second group includes proteins involved in signal sensing and signalling such as transcription factors, RNA-binding proteins, protein kinases, and phosphatases.

In this context, the ASRs [abscisic acid (ABA), stress, ripening] are the perfect example of a functional duality, dealing with both these types of stress-regulated proteins (Yang et al., 2005). On the one hand, the ASRs may be classified as LEA (late embryogenesis abundant) proteins, forming group 7 of this family, as recently proposed (Battaglia et al., 2008). Their affiliation to hydrophilins is mainly due to their common physiochemical features, with a high hydrophilicity index related to the important glycine content (Garay-Arroyo et al., 2000). Although the ASRs share some similarities with LEA proteins, they lack the LEA-specific signatures (Wise and Tunnacliffe, 2004). On the other hand, their DNA binding ability in a sequence-specific and Zn\(^{2+}\)-dependent manner through the transition from a disordered to an ordered state (Kalifa et al., 2004a; Goldgur et al., 2007; Maskin et al., 2007) is consistent with their behaviour as transcriptional regulators (Cakir et al., 2003; Shkolnik and Bar-Zvi, 2008). Moreover, some LEA proteins are also DNA-binding proteins (Wise and Tunnacliffe, 2004; Battaglia et al., 2008), but some other authors do not include the ASRs in the LEA family (Hundertmark and Hincha, 2008; Hunault and Jaspard, 2010). Finally, the preferential subcellular localization of the ASRs is not clear-cut between the cytoplasm (Kalifa et al., 2004a; Urtasun et al., 2010) and the nucleus.
(Cakir et al., 2003; Wang et al., 2003), and this location in two different cellular compartments further emphasizes their apparent dual role. However, the biological functions of these proteins remain unknown.

The main difficulty in the understanding of the physiological roles of these proteins is due to the fact that despite the characterization of at least 30 ASR genes in ~20 species, no homologous gene is found in the Arabidopsis genome. Furthermore, it seems that ASR genes are also missing from the Arabidopsis-related species Thellungiella halophilla (Wong et al., 2006) and the crucifer hyperaccumulator Thlaspi caerulescens (Plessl et al., 2005), leading to the suggestion that the Brassicaceae family possibly lacks their orthologues.

The responsiveness of ASR genes to distinct environmental cues, drought, cold, and osmotic stress, is studied mainly by RNA analysis. A restricted number of studies deal with ASR genes to distinct environmental cues, drought, cold, and osmotic stress, is studied mainly by RNA analysis. A restricted number of studies deal with ASR genes to distinct environmental cues, drought, cold, and osmotic stress, is studied mainly. (Rossi et al., 2003; Cakir et al., 2006) and the crucifer hyperaccumulator Thlaspi caerulescens (Plessl et al., 2005), leading to the suggestion that the Brassicaceae family possibly lacks their orthologues.

The responsiveness of ASR genes to distinct environmental cues, drought, cold, and osmotic stress, is studied mainly by RNA analysis. A restricted number of studies deal with the transcriptional regulation of ASR gene expression by means of promoter–reporter gene fusions. For example, it has been demonstrated that the potato ci21A promoter conferred cold responsiveness to the GUS (β-glucuronidase) reporter gene in the tubers of transgenic potato plants (Schneider et al., 1997). Furthermore, evidence was provided for the induction by ABA of tomato ASR2 promoter-controlled expression, after transient or stable transformation of two heterologous species, papaya and tobacco, but not in the homologous system, tomato and potato transgenic plants (Rossi et al., 1998). A larger study was conducted using two promoter regions of the potato StDS2 gene, belonging to the ASR family. The expression conferred to the GUS reporter gene was not significantly modified by a large panel of abiotic stresses (cold, heat, oxidative, and salt) and not even by exogenous ABA. However, the GUS activity was strongly induced by drought and treatment with 20% polyethylene glycol (PEG), demonstrating the importance of the proximal promoter region in the response to dehydration (Doczi et al., 2002).

In plants, the sugar status modulates and coordinates the integration of endogenous signals and environmental cues that govern growth and development. Previously, it has been demonstrated that the grape ASR, named VvMSA, is involved in the transcriptional regulation of the gene expression of the glucose transporter VvHT1 (Atanassova et al., 2003; Cakir et al., 2003). In the present study, several genomic VvMSA fragments are isolated from two grapevine varieties, and their polymorphism is demonstrated as arguing in favour of the presence of at least three alleles of the grape ASR gene. The functional analysis of the two most divergent VvMSA promoter regions reveals the conservation of a convergent hormonal (ABA) and metabolic (glucose) control of their reporter gene expression. Transient expression assays using the double luciferase system allow the targeting of the cis-acting motifs involved in this responsiveness. The preferential expression conferred by the VvMSA promoters in stems and petioles is demonstrated and is further shown to be confined to the phloem complex cells, both by stable transformation of tobacco and in situ hybridization in grapevine. Finally, the transcriptional regulation of grape ASRs expression is discussed in terms of the ABA and glucose signalling in the plant responses to environmental cues.

Materials and methods

Culture conditions of grape cell suspension and grape plantlets

The grape berry cell suspension derived from berries of the variety Cabernet-Sauvignon (CSB) was maintained at 24 °C on an orbital shaker (100 rpm) by weekly subculture in CSB medium (Decendit et al., 1996). Grape plantlets of the variety Ugni Blanc were micropropagated in Murashige and Skoog culture medium, at 24 °C during the day and 18 °C during the night, with 16 h light, at 250 μE m⁻².

Cloning of grapevine ASR promoter sequences

Genomic DNA was extracted from suspension-cultured cells for the variety Cabernet-Sauvignon (CS), and from leaves of grape plantlets propagated in vitro for the variety Ugni Blanc (UB). Freshly harvested grape cells or leaves from grape plantlets were frozen in liquid nitrogen, and conserved at −80 °C. Homogenized cells (1 g wet weight) were resuspended with 5 ml of the extraction buffer [200 mM TRIS-HCl, pH 9.0, 200 mM EDTA, pH 9.0, 1.5% SDS (w/v), 10 μM [β-mercaptoethanol] pre-heated at 60 °C. The mix was sequentially treated with RNase A (1 mg ml⁻¹) at 37 °C for 30 min and proteinase K20 (120 mg ml⁻¹) at 65 °C for 1 h, before phenol/chloroform purification and precipitation. Genomic DNA from 300 mg of homogenized grape leaves was extracted with 1 ml of a solution (2% cetrimethyl ammonium bromide (CTAB) (w/v), 2% polyvinylpolypyrrolidone (w/v)) by incubation at 65 °C for 15 min. After phenol/chloroform purification and precipitation, the DNA resuspended in water was treated with RNase A and proteinase K, as described before.

The isolation of the genomic fragments was performed with the BD GenomWaker System Universal kit (BD Biosciences) following the manufacturer’s recommendations. The primer sequences used, presented from the 5’ to the 3’ end, and the introduced restriction sites, in bold, were: for the genome walking GP31-TGGTGCACTTCCTCCTCCTCTGTAGTCA and GSP2-GTGTCCAGTGAAGTTGTTGTCGAGA; for the deletion of promoters, the forward primers ATGTTAGGTGGTGGTAAAGTTTGA and GSP2-GTGTCCAGTGAAGTTGTTGTCGAGA; and for internal primers for the complete sequencing, the forward primer ACGGATCCATACTAGCTGTA and the reverse primer AGACCATCGTTAAATTCTCTCTTCGAA (BanHI); and for internal primers for the complete sequencing, the forward primer ATGTTAGGTGGTGGTAAAGTTTGA and the reverse primer AATGAGGTTTTGCAGATGG and CTCTTGTGAGAGACCTACG. The VvMSA promoter sequences have been submitted to GenBank, and their accession numbers are: JN400118 for pVvMSA-CS, JN400120 for pVvMSA-CS/UB, and JN400119 for pVvMSA-UB.

In silico analysis and deletions of VvMSA promoters

In silico analysis of VvMSA promoter sequences was performed using the PLACE database (http://www.dna.afrc.go.jp/PLACE-fasta.html; Higo et al., 1999). The progressive deletions from the 5’ to the 3’ end of the promoters were produced using the naturally present restriction sites at the 5’ end (SacII from pGEM T-easy filled-in with Klenow, DraI, Rsal, XhoII, and HaeIII for the VvMSA-UB truncated promoters; Neol of pGEM T-easy, KpnI introduced by PCR and filled-in with Klenow, EcoRV, XhoII, and HaeIII for the VvMSA-CS promoters), and the PCR-introduced BamHI site at the 3’ end of both promoters. The obtained fragments of the 5’ regions were all inserted by directional cloning in the EcoRI/BamHI sites of the binary vector pBIN19-GUS used for the stable transformation of tobacco plants. The transient expression in grape protoplasts was performed using the appropriate plasmids, pluc carrying the firefly luciferase and pRLuc carrying
the Renilla luciferase under the control of p35S (Horstmann et al., 2004), a kind gift of Dr Eva Decker (University of Freibourg, Germany). The VvMSA-UB and VvMSA-CS deleted promoters were integrated into the pluc vector preferentially in the Smal–BanHI sites, with the exception of two fragments, the deletions 1 and 2 of pVvMSA-CS, which were inserted, respectively, in Smal–NcoI and Kpnl–BanHI sites. The 10 constructs pVvMSA::Firefly luciferase, corresponding to the five deletions for each promoter, were carefully verified by sequencing.

Protoplast isolation

Two days after subculture, grape cells were transferred into plasmolysis solution (CSB medium with 58 mM sucrose) containing the enzymes Cellulase Onozuka R-10 0.25% (w/v) and Macerozyme R-10 0.05% (w/v) (Duchefa, The Netherlands), for 16 h at 24 °C in the dark, under gentle shaking. After filtration through a 100 μm nylon mesh, the filtered suspension was overlaid on W5 medium (154 mM NaCl, 125 mM CaCl2, 5 mM KCl, 5 mM glucose), and the protoplasts were recovered by aspiration at the interface between these two phases. They were purified by three washes with W5 medium and centrifugation at 100 g for 10 min at room temperature. The protoplast pellet was resuspended in 10 ml of MIMM medium (15 mM MgCl2, 0.4 M mannitol, 0.1% MES, pH 5.6) for counting, and the final concentration was adjusted with the same medium to 1.7×106 protoplasts ml−1.

Transient transformation of CSB protoplasts

To each aliquot of 300 μl of protoplasts at a final concentration of 1.7×106 protoplasts ml−1 were added 21 μg of pVvMSA::Firefly luciferase, 6 μg of p35S::Renilla luciferase, 55 μg of carrier salmon DNA, and 300 μl of PEG solution [40% PEG 6000, 0.4 M mannitol, 0.1 M Ca(NO3)2, pH 8.0]. The transformation sample was carefully mixed before incubation for 30 min at room temperature, and subsequent gradual dilution with 10 ml of W5, slowly for 20 min. After centrifugation, the protoplasts were resuspended in 2.5 ml of CSB culture medium supplemented with 0.4 M glucose and 0.4 M mannitol, and placed at 24 °C, in the dark, for 24 h. For glucose depletion, the culture medium was supplemented with 0.8 M mannitol to maintain the same osmotic pressure. To avoid the stress due to protoplast isolation, 24 h later exogenous ABA at a final concentration of 10 μM was added to each of these media, thus creating the four tested culture conditions.

Luciferase assay

The CSB protoplasts co-transformed with the chimeric genes pVvMSA::Firefly luciferase and p35S::Renilla luciferase were harvested 48 h after their isolation (~24 h after the ABA treatment) by centrifugation at 100 g for 5 min and, after discarding the supernatant cells, were frozen in liquid nitrogen and stored at −80 °C. The frozen pellet was homogenized using a micropestle, centrifuged at 300 g for 30 s, and resuspended in 150 μl of the lysis buffer [100 mM KPO4 buffer, pH 7.8, containing 1 mM dithiothreitol (DTT)]. The measurement of the enzyme activities was performed by the Dual-Glo™ Luciferase Assay System according to the manufacturer’s recommendations (Promega, USA), and the light emission was read with a microplate luminometer ‘LumiCount’ (Packard, USA). The relative luciferase activity was calculated as the ratio between the firefly luciferase and the control Renilla luciferase activity. The transient expression assay was repeated independently 3–6 times for each construct with all samples measured in triplicate.

RNA gel blot analysis

Total RNAs were extracted from distinct grape organs (petioles, stems, leaves, and roots) of in vitro cultured UB plantlets using the method described by Valtaud et al. (2009). Equal amounts of RNA samples were separated by formaldehyde–agarose gel electrophoresis, transferred to Hybond N membranes (Amersham Life Science), and hybridized with the randomly primed [32P]VvMSA cDNA probe.

GUS fluorometric assay and histochemical test

The GUS fluorometric assay was performed on the different organs of tobacco transgenic plants according to the method of Jefferson et al. (1987), and the fluorescence emission was measured using a microplate fluorometer ‘FluoroCount™’ (Packard, USA). GUS histochemical stainings were performed by overnight incubation in the substrate solution [100 mM NaPO4 buffer pH 7.0, containing 0.5 mM potassium ferricyanide, K3Fe(CN)6, 0.5 mM potassium ferrocyanide, K4Fe(CN)6, 0.1% Triton X-100, 10 mM EDTA, and 0.5 mg ml−1 X-GlA (Duchefa, The Netherlands)]. The bright field observation and the acquisition of the whole germinations and hand-made cross-sections were done using a LEICA MZ 16 FA stereomicroscope (Germany). The thin sections (40 μm) were produced with a HBM 650V MICROM vibratome (Germany) after inclusion in 8% agarose, and were observed with a ZEISS axioplan (Germany) transmitted light microscope.

In situ hybridization

In situ hybridization was carried out on the leaves and petioles of the variety UB. The samples were fixed in a mixture of 4% paraformaldehyde and 0.5% glutaraldehyde in phosphate-buffered saline (PBS; 10 mM NaPO4, 145 mM NaCl, pH 7.2), embedded in paraffin for the preparation of 8 μm thin cross-sections, and further mounted on poly-l-lysine-coated slides. As the VvMSA cDNA was cloned in the pKSII plasmid (Stratagene), in the orientation 5′-SalI and 3′-NotI (Cakir et al., 2003), the sense and the antisense probes were produced after linearization of the vector with the restriction enzymes NotI and SalI, respectively. The synthesis of the dioxygenin-labelled riboprobes and the hybridization were performed as previously described in detail by Vignault et al. (2005).

Results

Cloning and polymorphism of VvMSA promoters

Gene sequences corresponding to VvMSA cDNA were isolated through the genome walking approach using genomic DNA extracted from two different cultivars UB and CS. The genomic fragments obtained allowed amplification of three distinct promoter sequences: (i) the first is specific to the cultivar CS and is named VvMSA-CS; (ii) the second belongs to the cultivar UB and is named VvMSA-UB; and (iii) the third is found in both studied cultivars, and is identified as an intermediacy sequence named VvMSA-CS/UB. The alignment of these three VvMSA genomic sequences allowed the demonstration of a complex polymorphism of grape ASR genes (Fig. 1A).

Previously, the VvMSA-CS cDNA was isolated with a pair of primers deduced from VvMSA-UB cDNA (Saumonneau et al., 2008), the latter cloned by the one-hybrid approach (Cakir et al., 2003). Like the vast majority of studied ASR genes, all VvMSA genes display two exons and one intron at ~100 bp, rich in A/T, and located at (+283 bp) or (+298 bp) downstream of the ATG codon (depending on the gene type). Interestingly, one repeated
Fig. 1. Comparison of the identified VvMSA promoters. (A) General structure of the VvMSA genes and schematic presentation of the main differences in the three cloned promoter regions. (B) Alignment of the three VvMSA promoter sequences: pVvMSA-CS, pVvMSA-CS/UB, and pVvMSA-UB. The insertions/deletions and substitutions found are indicated on the sequences by coloured boxes: the AATTCATTCCAATCAAGTC insertion/deletion in light blue; the GCGT/ATTA substitution in violet; the GAAT deletion/insertion in green; the CCCACCA insertion/deletion in orange; the CAAT-box in pink, the TATA-box in red, the transcription initiation site in red; the translation initiation site in dark blue; the single nucleotide polymorphism in green, red, blue, or yellow depending on the base T, A, C, and G, respectively; the unique deletion in the first exon is boxed in pink; and the single intron is indicated by a black hatched box. The primer sequences used for the amplification of the promoter regions are indicated by the red arrows.
sequence (AACGCTACTCCGAC) between positions 78 and 92 in the first exon is conserved in the clones UB and UB/CS, but is lacking in clone CS, corresponding to a deletion of 15 bp in the coding region and of five amino acids in the encoded protein, as already described by Saumonneau et al. (2008).

In addition to this unique deletion/insertion present in the first exon, the three VvMSA promoters display a most important polymorphism (Fig. 1B). Namely, the comparison of these 5′ regulation regions reveals that the VvMSA-CS promoter has at least three insertions/repetitions which are lacking in the VvMSA-UB promoter. The first insertion corresponds to the sequence AATTCATTCCAATCAA-GTC at position –1036 and the second one concerns the sequence GAAT at position –428, both encompassing potential sites for the fixation of the SUgar SIgnaling in Barley 2 (SUSIBA2) transcription factor responsible for a positive response to sucrrose (Sun et al., 2003, 2005). The third insertion is a repetition of the sequence CCCACCA located downstream to the TATA-box. In addition, the sequence GCGT conserved in the VvMSA-UB and the VvMSA-UB/CS 5′ regions is substituted by an ATTA sequence in the VvMSA-CS promoter, thus creating a potential AMYBOX1 motif (TAACAAA), conserved in α-amylase promoters as an essential element of negative regulation by sugar (Huang et al., 1990). The VvMSA-UB/CS promoter shares homologies with both VvMSA-UB and VvMSA-CS upstream regulatory sequences. Like the VvMSA-UB 5′ region, the CCCACCA sequence is not repeated and the GCGT sequence is conserved in the VvMSA-UB/CS promoter. In contrast, the VvMSA-UB/CS promoter bears, as does the VvMSA-CS 5′ region, the AATTCAATTCCAATCAA-GTC and the GAAT sequences.

**Table 1.** Sugar- and ABA-responsive motifs in silico found in the VvMSA promoter sequences.
WBOXATNPR1, WBOXTERF3, WRKY71OS, and WBOXPCWRKY1, putatively required for sugar induction. In addition, several cis-elements are known for their involvement in the hormonal control of gene expression (EBOXBNAPPAMYCCONSENSUSAT, MYCATERD1, MYCATRD22, GAREAT, ERELEE4, MYBIAT, and MYBGAHV). Interestingly, all these motifs are considered to be possibly implicated in the plant responses to environmental cues.

It is important to emphasize the complex redundancy of cis-elements, partly visualized by the common colours for different groups of motifs in Table 1. For example, AMYBOX1, GAREAT, and MYBGAHV are represented by the same consensus sequence (in plumb), clearly confirmed by their function and the copy number found in the VvMSA-CS promoter as well in the VvMSA-UB promoter. Another group concerns the drought response elements LTRECORATCOR15 and DRECRTCOREAT, where the latter consensus encompasses the first one (in green). A third case is displayed by the ABA motifs MYCATERD1 and MYCATRD22, which each correspond to a different strand of the same palindromic sequence, and both have the EBOXBNAPP consensus (light brown). Finally, the motifs for sugar induction (in red) are grouped around the most confirmed motif WBOXHVISO1, and all carry the consensus TGAC. However, no redundancy is observed for PYRIMIDINEBOXOSRAMY1A, CGACGOSAMY3, and MYB1AT elements.

The studied motifs are designed at their positions on the 5′ region sequences relative to the start codon (Fig. 2). The sugar response elements are spatially dispersed, covering the proximal, the central, and the distal part of the two VvMSA promoters. In contrast, the ABA response motifs are concentrated at the distal and the proximal promoter parts. Despite the high similarity, there is not a perfect identity between the respective motifs in terms of their exact position in the two promoter regions, as well as in terms of their copy number. As a matter of fact, the pVvMSA-UB 5′ sequence appears less abundant in terms of cis-elements than that of pVvMSA-CS. Moreover, this quantitative discrepancy concerns mainly the sugar response-related cis-elements, and the ratio is always in favour of the pVvMSA-CS promoter rather than the pVvMSA-UB promoter.

Deletions of VvMSA-CS and VvMSA-UB promoter regions: To complete the functional analysis, five progressive deletions were made from the 5′ to the 3′ end for each of the promoters, corresponding respectively to: CS1, 1193 bp; CS2, 756 bp; CS3, 566 bp; CS4, 259 bp, and CS5, 150 bp for pVvMSA-CS; and UB1, 1166 bp; UB2, 745 bp; UB3, 674 bp; UB4, 253 bp, and UB5, 144 bp for pVvMSA-UB (Fig. 3A, B). The deletions were produced in a manner
which allowed the sequential discrimination of different cis-elements. Concerning the \(VvMSA-CS\) promoter region (Fig. 3A), the progressive loss of motifs can be summarized as follows: for the ‘full-length’ CS1 construct, there are 11 ABA and 11 sugar motifs, the latter corresponding to eight elements for sugar repression (S–) and three elements for sugar induction (S+); for CS2 deletion, there are seven ABA- and nine sugar-responsive motifs (7 S– and 2 S+); for CS3 deletion, there are seven ABA and six sugar consensus sequences (4 S– and 2 S+); and for the CS4 deletion, there are three ABA and two sugar elements (1 S– and 1 S+). The CS5 deletion was completely depleted of the motifs studied.

In parallel, the analysis of targeted motifs of the \(pVvMSA-UB\) region (Fig. 3B) revealed that: the ‘full-length’ UB1 sequence carried 11 ABA and eight sugar response motifs (7 S– and 1 S+); the UB2 deletion encompassed six ABA and six sugar consensus sequences (5 S– and 1 S+); the UB3 deletion encompassed six ABA and five sugar elements (4 S– and 1 S+); and, finally, the UB4 deletion had only one conserved ABA consensus sequence, and this was lost by the UB5 construct, corresponding to the minimal promoter.

All these truncated promoters are transcriptionally fused in front of the coding region of the reporter gene, thus producing five chimeric constructs of the \(VvMSA\) promoter. To increase the reliability of the expression regulation study, the Dual-Glo Luciferase Assay System (Promega) which assays both the firefly (\(Photinus pyralis\)) luciferase and the \(Renilla\) (\(Renilla reniformis\)) luciferase was applied (Horstmann et al., 2004).

The analysis was realized by co-expression of the studied \(pVvMSA::Firefly luciferase\) gene and the \(p35S::Renilla luciferase\), as an internal control for the efficiency of transformation of grape protoplasts. Additional control constructs

Fig. 3. Functional analysis of \(VvMSA\) promoters by transient transformation of CSB cells with firefly luciferase as a reporter gene. Each of the five deletions of (A) \(pVvMSA-CS\) and (B) \(pVvMSA-UB\) with their encompassed motifs is presented in front of the reporter gene conferred expression. The numbers above the deletions indicate their size in bp. The luciferase activity is presented by the RRR (the Report of the Relative Response) in arbitrary units. The bars correspond to the mean values of at least three independent biological repetitions, with three technical replicates for each (\(n\)=9), and the standard error is calculated.
were the promoterless: Firefly luciferase gene as a negative control and the constitutive promoter 35S CaMV: Firefly luciferase gene as a positive control.

**Comparison of the expression level conferred by truncated VvMSA promoters**

The chimeric constructs corresponding to pVvMSA-CS and pVvMSA-UB deletions fused to the firefly luciferase were simultaneously introduced (under standardized conditions of the same experiment) into grape protoplasts by PEG transformation. The expression conferred on the reporter gene was further analysed by quantification of enzyme activity, based on the luminescence emission (Fig. 3A, B). However, it is important to emphasize the fact that all the deletions for each promoter were always studied in parallel in the same experiment for the independent biological repetitions, obviously including each three technical replicates. In contrast, the deletions of both promoters were never studied in the same experiment, because the aim was to observe the effect of different deletions corresponding to a given 5’ region.

To determine this, the deletions to each of the studied promoters were compared. The pVvMSA-CS-confferred expression, presented in Fig. 3A, diminished progressively between construct CS1 and CS2 (–15%), and CS2 and CS3 (–30%), but dropped drastically between the CS3 and CS4 deletions (–80%). This latter deletion showed nine and five times reduced activity compared with the constructs CS1 and CS3, respectively. However, the CS4 construct was able to control a certain level of expression before its final restriction to the basal level imposed by the CS5 deletion, corresponding to the minimal promoter. Thus, the level of pVvMSA-CS-confferred expression was gradually decreased in parallel with the reduction of the size of the 5’ region up to its almost total abolishment for the minimal promoter, encompassing the CAAT and TATA boxes.

The reporter gene expression conferred by pVvMSA-UB is presented in Fig. 3B. However, the progressive deletion of the studied motifs produced an unexpected decrease in luciferase activity controlled by the UB2 deletion, representing 40% of that conferred by UB1. The construct UB3 displayed an enhanced expression, nearly reaching that of the ‘full-length’ promoter (UB1). The last two deletions UB4 and UB5 were remarkable in the sharp reduction of reporter gene activity, becoming almost undetectable. In comparison with pVvMSA-CS truncated promoters, the decrease in the expression conferred by pVvMSA-UB deletions was not linear, and the drop of reporter gene activity for the last two deletions appeared most drastic.

**Responsiveness of VvMSA promoters to glucose and ABA signals**

The effects of glucose and ABA on the pVvMSA/Luciferase chimeric genes were analysed for the first three deletions produced for each of the VvMSA promoters: CS1, CS2, CS3, and UB1, UB2, and UB3, respectively. To study the responsiveness of VvMSA promoters to sugar and ABA signals, four different conditions were tested. The first was the normal culture medium, containing 0.4 M glucose as carbon source and 0.4 M mannitol as an additional osmotic agent. The second medium was depleted of glucose, and complemented only with mannitol at 0.8 M to maintain the same osmotic pressure. The third medium was the normal culture medium but supplemented with 10 μM ABA in the presence of glucose. The last was also supplemented with 10 μM ABA, but in a condition of glucose starvation.

The reporter gene activities obtained for each construct with the standard culture medium (violet bars) were considered as 100% and all other results were presented as values relative to this condition (Fig. 4A, B). As shown in this figure, the glucose depletion (green bars) increased the reporter gene activity conferred by the different constructs: CS1, 170%; CS2, 170%; CS3, 210%; UB1, 150%; and UB2, 360%. Such stimulation of expression due to the absence of glucose was not observed for the UB3 deletion, being the same as in the glucose-containing medium. Compared with the other CS and UB deletions, the UB2 construct showed the highest responsiveness to glucose depletion.

The addition of exogenous 10 μM ABA in the presence of glucose (orange bars) considerably induced the level of expression of all the studied promoters, the reporter gene activity ranging as follow: CS1, 180%; CS2, 250%; CS3, 430%; UB1, 160%; UB2, 180%; and UB3, 200%. This treatment revealed a strong ABA induction of both VvMSA promoters, further enhanced by the successive deletions studied, before the CS4 deletion, marked by a decrease of ABA effect, but still sufficiently ABA inducible (data not shown).

For the last condition, the addition of exogenous ABA in the absence of glucose (blue bars) produced differential responses of the distinct constructs. Thus, the expression conferred by the CS2, UB1, UB2, and UB3 constructs was strongly enhanced to 500, 200, 480, and 380%, respectively, suggesting a clear-cut synergistic effect of ABA and glucose depletion. However, the ‘full-length’ promoter CS1 displayed an increase in expression (165%) almost equal to that due to the absence of glucose in the medium that may indicate the loss of ABA induction. Moreover, the CS3 deletion also demonstrated a significant induction, more important than that produced by simple glucose depletion, but largely insufficient for a synergism between ABA and glucose starvation.

**Organ specificity of the expression pattern conferred by VvMSA promoters**

To study the expression specificity conferred by the VvMSA promoters, tobacco plants were transformed with the different chimeric genes corresponding to pVvMSA-CS and pVvMSA-UB deletions fused in front of the GUS reporter gene. At least three independent transgenic clones were analysed for three deletions of each of both promoters. In planta analyses were targeted to four distinct organs: petioles, stems, leaves, and roots. After PCR detection of chimeric transgenes in the genomic DNA of genetically modified tobacco, the reporter gene activity was measured.
by the GUS fluorimetric assay. Tobacco plants expressing the p35S::GUS construct were produced as a strong positive control, and wild-type tobacco served as a negative control. The level of GUS activity was almost 10-fold weaker than that controlled by p35S (data not shown), but still remained sufficient to confer correct expression under in vitro culture conditions. The detailed analysis of expression conferred by both VvMSA promoters revealed a strong preferential GUS activity in petioles and shoots (Fig. 5A, B). A less important level was detected in leaves. Finally a reduced and nearly undetectable fluorescence was demonstrated in roots for pVvMSA-CS and pVvMSA-UB, respectively. These results obtained in a heterologous system, transgenic tobacco plants, were further confirmed in the homologous system, namely grape plants (Fig. 5C). The RNA analysis in UB in vitro plantlets revealed a strong VvMSA-UB expression in petiole, a relatively high levels in stems and leaves, and a decrease in transcripts in roots.

To complete the study of expression specificity, the GUS histochemical test was applied. As presented in Fig. 6, the GUS activity controlled by both ‘full-length’ promoter regions was compared in the same four organs as described above, and the wild-type tobacco plants were used as a negative control. Detailing the results from the histochemical assay, it was obvious that the GUS expression conferred by the two VvMSA promoters appeared early at germination, and the young seedlings displayed decreasing blue staining from the stem apex, through the first true leaves, to the residual coloration of cotyledon cells (Fig. 6A–C). Interestingly, the young primary roots also showed a blue...
colour, namely in their upper parts below the root–stem junction. Furthermore, the GUS staining was the most pronounced in the tissues of petioles and stems, thus confirming the results from the fluorimetric test. The predominant blue colour in petioles corresponded to the conducting tissues and the cortical cell layers (Fig. 6D, E). In the stems, the GUS staining concerned mainly the elements of the conducting complex, while the blue colour was weaker in the stem cortical parenchyma (Fig. 6F–H). The stems and the petioles showed a more intense staining (Fig. 6F–H), and the latter two appeared as slightly blue shaded. Finally, a most precise observation realized on thin layer slices from stems demonstrated that the GUS expression was confined to the phloem complexes, the primary and the secondary complexes, as visualized for each of the promoters \( pVvMSA-CS \) and \( pVvMSA-UB \), respectively (Fig. 6O, P).

In situ hybridization visualization of \( VvMSA \) expression in grape

To analyse further in depth the cell type-specific expression of \( VvMSA \), in situ hybridization experiments were performed in leaves and petioles from the variety UB (Fig. 7A–F). The \( VvMSA \) transcripts were localized using a digoxigenin-labelled antisense riboprobe, covering the full-length cDNA of the \( VvMSA-UB \) gene. The specificity of hybridization was also checked with the corresponding sense riboprobe as a negative control. Despite the weak ASR expression level in mature leaves, the in situ hybridization revealed a preferential signal in the main vein when compared with the mesophyll cells (Fig. 7A, B). Moreover, visualization of the grape ASR transcripts clearly matched with the cells of phloem complexes (Fig. 7A, B). In addition, the in situ hybridization applied on the petiole cross-sections (Fig. 7C, D) confirmed that the blue staining was confined to the phloem of primary and secondary bundles, and was exclusively revealed with the antisense riboprobe only, which means in the positive test and not in the negative control. Magnification of the principal vein hybridization (Fig. 7E, F) allowed the localization of \( VvMSA \) expression in the internal phloem complexes. No signal was detected in the parenchyma, epidermis, and xylem cells for the \( VvMSA \) gene, whereas in parallel a control \textit{dehydroflavanol 4-reductase} (DFR) antisense riboprobe allowed hybridization in all these cell types (data already published, Vignault et al., 2005), thus confirming the preferential phloem-specific expression of the \( VvMSA-UB \) gene.

Discussion

The grape ASRs—alleles or genes?

The divergence in the function and the expression profile in a family of genes improves the plasticity and innovation, thus ensuring evolution of the species (Ohta, 1989). To date, the polymorphism of ASR genes has been studied in two species, tomato and rice (Frankel et al., 2003, 2006; Giombini et al., 2009). In tomato, there are four genes all located on the same chromosome IV, but not exclusively in the same locus. In contrast, in rice, the six ASR genes are localized on several chromosomes, mainly on chromosome I (Frankel et al., 2006). The ASR gene family of Zea mays contains nine genes, from \( ZmASR1 \) to \( ZmASR7-3 \), mapped to five of the 10 maize chromosomes, with chromosomes 2 and 10 encompassing two loci and chromosome 3 carrying three loci (Virlouvet et al., 2011).

The three described different versions of the grape ASR gene display a single deletion/insertion and only two mutations in the coding region (Fig. 1A, B). The unique deletion in the first exon concerns 15 bp corresponding to
five amino acids, and is located in a repeated region (Saumonneau et al., 2008). This means that even with excision of this sequence, the \( VvMSA-CS \) coding region is not completely depleted of it, due to its partial repetition in the N-terminal part of the encoded protein. Furthermore, a peptide encompassing the amino acid residues involved in

---

**Fig. 6.** GUS histochemical staining, corresponding to the expression conferred by the full-length promoters (CS and UB) in the different organs of wild (WT) and transgenic tobacco plants: germinations (A–C); petiole cross-sections (D and E); stem cross-sections (F–H); roots (I–K); leaves (L–N); stem thin cross-sections (O and P).
the deletion allowed a polyclonal antibody to be raised against the VvMSA-UB full-length protein, but it also perfectly recognized the VvMSA-CS truncated protein, thus sharing a high specificity for grape ASR (Saumonneau et al., 2008).

Previously, an *in silico* analysis of eight tentative consensuses corresponding to 1353 expressed sequence tags (ESTs) from different grape varieties (http://compbio.dfci.harvard.edu/tgi/plant.html) showed the presence of the two ASR proteins in Cabernet-Sauvignon, Chardonnay, and Shiraz. In contrast, only the full-length form was identified in Ugni Blanc and only the truncated protein was present in Pinot Noir. These data suggest that the grape ASR proteins may be represented by one of the forms, full length or truncated, or by both of them (Saumonneau et al., 2008). In this context, the BLAST analysis of the *VvMSA* gene sequence against the proteome 8× deduced from the Pinot Noir genome (Jaillon et al., 2007) reveals identity within a unique locus.
(GSVIVP 00031749001). Furthermore, the plant material used in the present experiments (UB in vitro plantlets, as well CS cells in culture) was monoclonal and heterozygous. All these considerations suggest the existence of VvMSA alleles and provide plausible arguments in favour of the allelic polymorphism of the grape ASR gene. Moreover, the high number of single nucleotide substitutions and the deletions/insertions, repetitions, and mutations are preferentially concentrated in the promoter regions. Finally, the polyallelic character of the VvMSA gene perfectly matches the definition of gene alleles described as distinct versions of the same gene, displaying differences in their nucleotide sequences due to mutations during evolution, and all regrouped into a single locus of the same chromosome. A confirmation of this sequence-based assumption may be provided by the functional study of 5' regions involved in gene expression regulation.

Phloem co-localization of VvMSA and VvHT1 transcripts

The study of the organ specificity conferred by the two VvMSA promoters in transgenic tobacco plants reveals their preferential expression in the petioles and the stems, compared with the leaves and the roots (Figs 5A, B, 6A–F). The highest level in petioles and the lowest level in roots are further confirmed by RNA analysis in grape plantlets (Fig. 5C). In grapevine, using an in situ hybridization approach, it was demonstrated that grape ASR gene expression is confined to the conducting complex of the phloem (Fig. 7). This expression pattern of grape ASR is in accordance with the results reported for the tomato ASR1, primarily observed in the phloem companion cells (Maskin et al., 2008), which is not contradictory to the expression detected in bundle sheath cells of sugarcane (Suguiharto et al., 2003). Furthermore, these findings are in good agreement with the localization of the expression of the hexose transporter gene VvHT1 (Vignault et al., 2005), the target of the transcription factor encoded by the studied VvMSA gene, also detected in phloem companion cells. The co-localization of both of these molecular partners in the conducting complex further confirms the role of VvMSA in the transcriptional regulation of the VvHT1 hexose transporter gene (Cakir et al., 2003).

Glucose and ABA signalling in the control of VvMSA expression

In a general manner, the reported results demonstrate that both VvMSA promoters are responsive to glucose and ABA treatments, and the combination of both effectors may display some synergistic effects (Fig. 4A, B). pVvMSA-CS encompasses a very important number of sugar- and ABA responsive cis-elements (11 sugar motifs and 11 ABA motifs), compared with pVvMSA-UB carrying eight sugar and 11 ABA motifs. Interestingly, the number of motifs for sugar induction and sugar repression differs between them, as, for example, the WBOXVIS01 element for sugar induction and the AMYBOX1 element for sugar repression, with a ratio of 3/1 and 2/1 for pVvMSA-CS versus pVvMSA-UB, respectively. This allows speculation that when compared with pVvMSA-UB the higher responsiveness of pVvMSA-CS to the complex cross-talk of glucose and ABA signalling seems to be mainly influenced by the sugar-related motifs.

In this context and under glucose starvation (Fig. 4A, B, green bars), the deletion UB2 demonstrates the most important induction, and, accordingly, the same UB2 deletion undergoes the most pronounced inhibition by glucose (Fig. 3B). These results corroborate the suggestion that among the studied constructs the UB2 deletion seems to have the greatest responsiveness to glucose. In contrast, the ‘full-length’ promoter UB1 and the UB3 deletion both confer a quite similar high level of expression in the presence of glucose, and are slightly or not affected by glucose starvation. A careful analysis of the sugar repression/induction elements between the UB1, UB2, and UB3 promoters reveals the progressive deletion of the PYRIMIDINEBOXOSRAMY1A motifs for each successive truncated construct. Interestingly, the suppression of the promoter sequence between UB2 and UB3 (from −745 bp to −674 bp), encompassing the third PYRIMIDINEBOXOSRAMY1A, completely abolishes the induction due to glucose depletion observed for UB2, and UB3 seems not to be repressed by glucose. This promoter fragment of only 71 bp appears to be required for the repression of UB2 by glucose. It is noteworthy that none of the produced pVvMSA-CS deletions demonstrates such elevated susceptibility to glucose repression or such induction under glucose depletion (Figs 3A, 4A). This apparent discrepancy in the glucose response of CS2 and UB2 deletions, whose sizes differ by only 11 bp, seems not to be directly related to the number of sugar-responsive elements, but may mostly reflect their differential combinations in this important promoter region.

A first synergistic effect is observed for ABA in the presence of a high concentration of glucose (Fig. 4A, B, orange bars). Moreover, the level of ABA induction of grape ASR expression is also enhanced by the different truncated promoters, CS2, CS3, UB2, and UB3, before decreasing for CS4 (data not shown). In fact, between CS3 and CS4, the proximal promoter has lost three additional sugar repression

Fig. 8. Model for glucose and ABA signalling in VvMSA transcriptional regulation. Details are explained in the text.
motifs and one sugar induction motif, thus reaching a ratio of 1 for sugar-positive and negative elements. In parallel, the CS4 deletion conserves three consensus sequences responsive to ABA, and between them two copies of the MYBAT1 and one of the DRECRTTCOREAT motifs. The ABA-inducible expression of CS4 seems completely lost in the case of the UB4 deletion, which never displays any detectable activity conserving only the last DRECRTTCOREAT box in the absence of any sugar-responsive motif. Taken together, these results suggest that the MYBAT1 elements have a crucial role for ABA responsiveness under high glucose concentration, requiring the presence of at least one sugar induction motif, such as WBOXHVIS01.

Secondly, the ABA induction is observed under glucose starvation for the CS2, UB1, UB2, and UB3 promoters (Fig. 4A, B, blue bars). In addition, this positive responsiveness is enhanced by the deletion of the distal part of the \( V_{vMSA} \) 5' regulatory regions, for example the deletions CS2, UB2, and UB3 for which the expression is increased up to 3-, 2.4- and 1.9-fold, respectively, in comparison with the ‘full-length’ promoters. The critical deletion for the synergistic effect between glucose depletion and ABA appears to be CS3, which eliminates three sugar repression motifs and changes the ratio of the sugar repression/sugar induction elements from 3.5 for the CS2 construct to 2.0 for the CS3 construct.

In terms of the role of sugar signalling on the \( V_{vMSA} \) promoters, a first critical element to be taken into consideration is the concentration of glucose in the protoplast culture medium. In fact, the integrity of isolated protoplasts is maintained in an isotonic medium by 400 mM glucose and 400 mM mannitol as an additional osmotic reagent. This elevated concentration of glucose is much higher than that required for the activation of its cytosolic sensor the hexokinase HXK1, with a concentration of 150 mM considered as sufficient to repress the expression of the target of \( V_{vMSA} \) transcription factor, the glucose transporter gene \( V_{vTH1} \) (Conde et al., 2006). Interestingly, even at a high glucose level the activity conferred by \( V_{vMSA} \) promoters is not completely abolished, which may suggest a certain HXK1 independence of \( V_{vMSA} \) expression. This observation is in good agreement with the role of \( V_{vMSA} \) in the transcriptional induction of \( V_{vHT1} \) gene activity at physiological concentration of glucose (56 mM) (Atanassova et al., 2003). Furthermore, in the model proposed by Conde et al. (2006) for glucose signalling in the regulation of expression of the hexose transporter gene \( V_{vHT1} \), there is one HXK1-dependent pathway for a high concentration of glucose and a second HXK1-independent pathway for a low concentration of glucose. The complex role of \( SISRI \) in glucose metabolism in the tubers of transgenic potato plants (displaying its overexpression or down-regulation) also confirmed this elusive perception concerning the biological function of ASR (Frankel et al., 2007).

Eventually, a plausible model for \( V_{vMSA} \) transcriptional regulation at the convergence of glucose and ABA signalling cascades may be proposed (Fig. 8). First, it may be supposed that in the normal protoplast culture medium the sensing of the high glucose concentration by HXK1 restricts \( V_{vMSA} \) expression to its HXK1-independent part (Fig. 4, violet bars). Secondly, the induction of \( pV_{vMSA} \) expression by glucose starvation may be suggested as the result of the suppression of the \( V_{vMSA} \) transcriptional inhibition due to HXK1, thus allowing the addition of this HXK1-dependent activity to the HXK1-independent activity, discussed above (Fig. 4, green bars). Furthermore, the enhancement of \( pV_{vMSA} \) expression by exogenous ABA, even in the presence of a high glucose concentration, may be explained by the additional effect of ABA signals, the exogenous ABA and the endogenous ABA, whose synthesis is induced by HXK1 as a glucose sensor (Fig. 4, orange bars). This strong ABA signalling appears to counteract and outweigh the HXK1 repression. Finally, the synergistic enhancement of \( pV_{vMSA} \) expression by ABA and glucose starvation (Fig. 4, blue bars) appears to be the result of three effects: exogenous ABA signalling, the inhibition of HXK1 repression, and the activation of SnRK1 (SnF1-Related Kinase 1) as a central regulator under energy deficit, glucose starvation, and darkness (Rolland et al., 2006; Baena-Gonzalez et al., 2007; Baena-Gonzalez and Sheen, 2008). This synergistic effect is strongly pronounced for the promoter deletions CS2, UB1, UB2, and UB3, but failed for CS1 and CS3 constructs. However, the responsiveness to the described pathways for \( V_{vMSA} \) differential regulation is dependent on the promoter deletions produced, dealing with their presence/absence and the real biological activity of these in silico identified cis-elements.

Previously, it was demonstrated that the stimulation of \( V_{vMSA} \) expression by exogenous ABA requires the presence of sucrose at a physiological concentration of 58 mM (Cakir et al., 2003). This effect is further extended to other disaccharides carrying a glucosyl moiety, and confirmed by palatinose, a non-hydrolysable and non-transportable sucrose analogue, providing the evidence that sucrose signalling needs a putative sensor located on the plasma membrane (Atanassova et al., 2003). The present results, concerning on the one hand, the synergistic effect of ABA and glucose starvation, and on the other hand, the possible effect of high glucose concentration on ABA synthesis, reveal the complexity of sugar signalling in the transcriptional control of grape ASR genes, and consequently emphasize the fine tuning of the \( V_{vMSA} \) target genes. Moreover, the involvement of tomato and lily ASR genes in the tolerance to water deficit and osmotic stress has already been demonstrated (Kalifa et al., 2004b; Yang et al., 2005). Taken together, these results argue in favour of a role for ASR in the internalization of the environmental signals.

To conclude, evidence is provided for the allelic polymorphism of the grape ASR gene, which is further confirmed by the phloem-conducting tissue-specific localization and the common \( V_{vMSA} \) promoter responsiveness to ABA and glucose. The present data and previous results on the convergence of sugar and ABA signalling in the control of \( V_{vMSA} \) and its target gene, encoding the glucose transporter \( V_{vHT1} \), strongly emphasize the complex role of grape ASR in the fine tuning of gene expression regulation by metabolic
and hormonal signals, involved in the integration of the environmental cues.

Acknowledgements

We are very grateful to Dr Eva Decker, University of Freiburg (Germany), for the kind gift of the plasmids carrying the firefly and Renilla luciferase genes. We thank Mr Gregory Guillot for his valuable help with the transient expression in grape protoplasts. We express our gratitude to Mrs Marie-Thérèse Bidoyen, who died in August 2008, for her excellent technical assistance and human qualities. This work was supported in part by a grant to AS from the Conseil Régional Poitou-Charentes.

References


Hundertmark M, Hincha DK. 2008. LEA (late embryogenesis abundant) proteins and their encoding genes in Arabidopsis thaliana. BMC Genomics 9, 118.


