FEBS Letters 582 (2008) 3281–3287

Interaction of grape ASR proteins with a DREB transcription factor in the nucleus

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Received 23 July 2008; revised 1 September 2008; accepted 2 September 2008

Available online 18 September 2008

Edited by Ulf-Ingo Flügge

Abstract ASR proteins (abscissic acid, stress, ripening induced) are involved in plant responses to developmental and environmental signals but their biological functions remain to be elucidated. Grape *ASR* gene (*VvMSA*) encodes a new transcription factor regulating the expression of a glucose transporter. Here, we provide evidence for some polymorphism of grape ASRs and their identification as chromosomal non-histone proteins. By the yeast two-hybrid approach, a protein partner of VvMSA is isolated and characterized as an APETALA2 domain transcription factor. Interaction of the two proteins is further demonstrated by the BiFC approach and the exclusive nuclear localization of the heterodimer is visualized.

Structured summary:
MINT-6743067: *VvMSA* (uniprotkb:Q94G23) and *VvDREB* (uniprotkb:A6XA90) *physically interact* (MI:0218) by *bimolecular fluorescence complementation* (MI:0809)
MINT-6743043: *VvMSA* (uniprotkb:Q94G23) *physically interacts* (MI:0218) with *VvDREB* (uniprotkb:A6XA90) by *two hybrid* (MI:0018)

Keywords: ASR proteins; DREB transcription factor; Protein-protein interaction; Yeast two-hybrid; BiFC; Grapevine

1. Introduction

Since the cloning of the first *ASR* (abscisic acid, stress and ripening induced) gene in tomato [1], more than 30 *ASR* genes have been identified in different plants species, but there is not an ASR gene found in the genome of the model plant *Arabidopsis thaliana*. The induction of their expression by diverse stresses – water deficit, cold, salt, limited light [2,3], the cloning of maize ASR as genes linked to drought resistance [4], and the divergence of tomato ASR paralogs due to the adaptation to a hostile environment [5] strongly suggest the role of these proteins in the plant response to environmental cues. ASR genes may also be involved in developmental processes, such as seed and pollen germination [6,7], leaf senescence [8] and fruit ripening [9,10].

By a yeast one-hybrid approach, using as target the proximal promoter of glucose transporter gene *VvHT1,* we isolated a grape *ASR* cDNA [9] named *VvMSA* (*Vitis vinifera* maturation, stress, ABA). The direct binding of VvMSA protein to the *VvHT1* promoter was shown in a gel shift assay and confirmed by in planta co-expression experiments. Thus, a molecular function was ascribed to an ASR protein, as a transcriptional regulator of a glucose transporter gene expression at the cross-talk of sugar and ABA signaling [9]. Recently, another ASR protein *ci21A*/*ASR1* was demonstrated to be involved in the regulation of glucose metabolism of potato tuber [11].

The low molecular weight and high hydrophilicity of ASRs may design them as putative candidates for direct protein protection, like some LEA proteins [2,10], furthermore their preferential nuclear localization [9,12] and direct interaction with DNA [13,9,12,14] suggest acting as transcriptional regulators of gene expression. However, the biological functions of these proteins remain unknown.

In this work, we demonstrate some polymorphism of grape ASRs at cDNA and protein level, and their identification as chromosomal non-histone proteins. Using yeast two-hybrid screening, we find a protein partner of grape ASR and characterized it as an AP2 domain transcription factor named VvDREB (*Vitis vinifera* drought response element binding protein). Interaction between the two proteins is demonstrated in grape cells by a bimolecular fluorescence complementation (BiFC) approach, and localized exclusively in the nucleus. These results design grape ASRs as transcription factors of architectural type, able to recruit another regulatory protein such as VvDREB.

2. Materials and methods

2.1. Plant material

The grape berry cell suspension derived from Cabernet Sauvignon berries (CSB) was cultured as previously described [9]. Grape plantlets 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}.

2.2. Cloning of VvMSA cDNA

The cloning of *ASR* cDNA from grape CSB cells was achieved by RT-PCR with *VvMSA* specific primers (5′-GACTAGTTTTGATCACCACCAC-3′; 5′-CCGGAATTCAACATCAAACTATAATC-3′) carrying the restriction sites SpeI and EcoRI, respectively.

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0014-5793/$34.00
doi:10.1016/j.febslet.2008.09.015

2.3. Production of anti-VvMSA antibodies

Two peptides (1: SDNAYSDTTYSDTSY; 2: SDTTYSDTSYATDGV) were chosen in the most specific sequence of VvMSA protein, and were used as immunogens to raise polyclonal monospecific antibodies in rabbits (Eurogentec, Belgium).

2.4. Nuclei isolation and fractionation of nuclear proteins

Nuclei were isolated from grape CSB cells and nuclear proteins were fractionated as previously detailed [15]. Nucleosolic proteins were extracted with 0.15 M NaCl in 10 mM Tris/HCl, pH 7.5, and the acid-soluble chromosomal proteins with 0.4 N H_2SO_4. The protein concentration was estimated by the bicinchoninic acid assay according to the manufacturer's instructions (Pierce, USA). Proteins were separated by tris–glycine SDS–PAGE on 16% gels.

2.5. Two-hybrid screening

The coding region of *VvMSA* was amplified by PCR with the specific primers (5′-ACGTCGACCATGTCGGAGGAGA-3′; 5′-CGACTAGTAGAAAAGATGGTGAT-3′) containing the restriction sites SalI and SpeI, respectively, and inserted in pDBLeu plasmid. The prey cDNA library from grape berry was produced in the pPC86 vector as described by Cakir et al. [9]. Yeast strain MAV 203 (Invitrogen, France) was co-transformed with both bait and prey, using a lithium acetate protocol.

2.6. Isolation of protoplasts

Two days after subculture, grape cells were transferred into plasmolysis solution (CSB medium with 0.4 M sucrose) containing 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. Protoplasts were harvested at the interface of 100 μm filtered suspension and superposed W5 medium (154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 5 mM glucose) and three times washed in W5 medium (centrifugation for 10 min at $100 \times g$, at room temperature). The protoplast pellet was suspended in MMM medium (15 mM $MgCl_2$, 0.4 M mannitol, 0.1% MES, pH 5.6) at a final concentration of 1.7×10^6 protoplasts per mL.

2.7. Transient expression

Protoplasts were incubated for 30 min on ice and to 300 μL of suspension (5×10^5 protoplasts) were added 20 μg of plasmid DNA for each construct, 80 μg of Salmon DNA, 300 μL of PEG solution

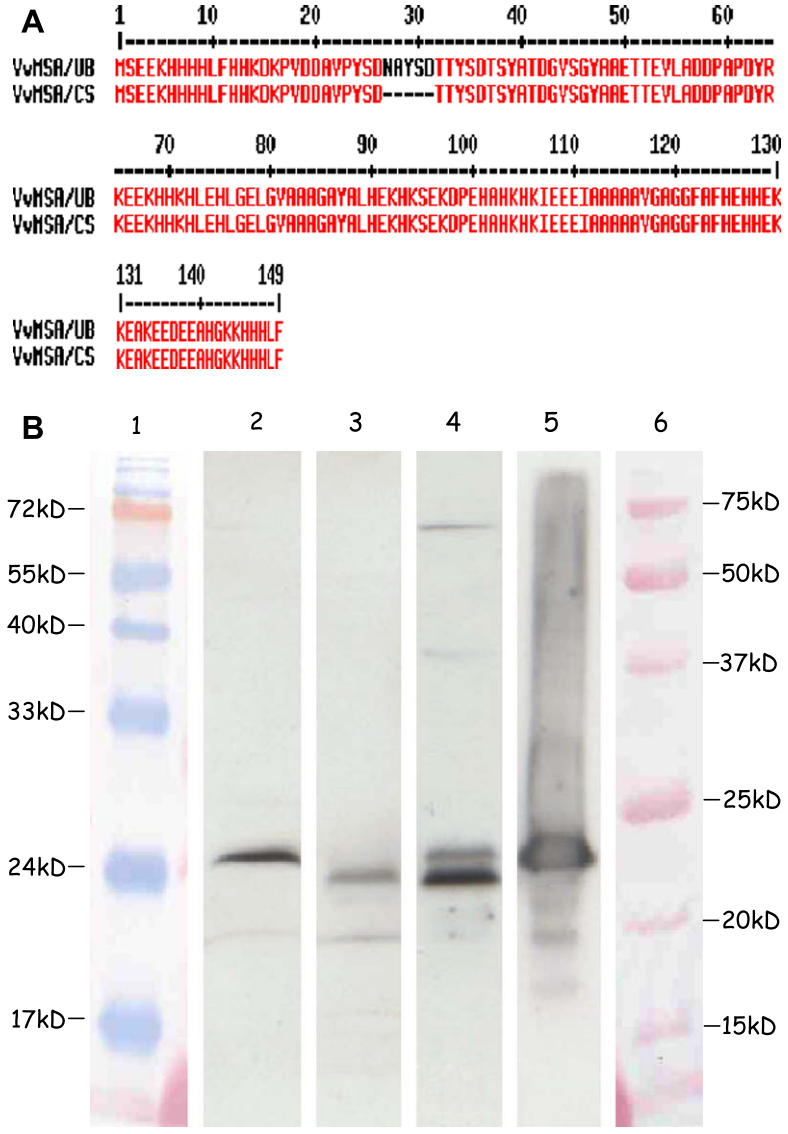

Fig. 1. Evidence for existence of two VvMSA proteins. (A) Alignment of VvMSA proteins (deletion is dashed). (B) Visualization of VvMSAs: 1 and 6. Protein markers; 2 and 3. TnT-produced proteins (4 μL of the reaction); 4 and 5. Acid-soluble proteins from CS and UB varieties (20 μg). Immunodetection with specific anti-VvMSA antibody (1/1000).

(31% PEG 6000 (v/v), 0.4 M mannitol, 0.1 M $Ca(NO_3)_2$, pH 8.0). Protoplasts were allowed to rest for 30 min at room temperature and diluted slowly to 10 mL with W5 medium, and finally suspended in 2.5 mL of CSB modified medium (0.4 M glucose and 0.4 M mannitol) for 48 h culture at 24 °C in the dark, before observation.

2.8. Confocal imaging

A spectral confocal station FV1000 installed on an inverted microscope IX-81 (Olympus, Tokyo, Japan) was used and images were acquired with an Olympus UplanFLN ×60 water, 1.2 NA, objective lens resulting in 512 × 512 pixels images with 0.14 μm/pixel. YFP was excited with argon laser at 515 nm and the emitted fluorescence was detected in a channel between 530 and 630 nm.

3. Results

3.1. Polymorphism of VvMSA proteins

Only one *VvMSA* cDNA was isolated corresponding to a single gene detected by Southern blot in the genome of the grape variety Ugni blanc (UB) [9]. The cloning of another *ASR* cDNA from grape variety, Cabernet-Sauvignon (CS), revealed the lack of 15 bp in the coding region leading to a deletion of five amino acid residues, namely NAYSD, in the VvMSA-CS sequence (Fig. 1A).

To check the presence of two different proteins in studied varieties, the cDNAs, *VvMSA-UB* and *VvMSA-CS,* were expressed in the TnT® Coupled Reticulocyte Lysate System (Promega, USA), and the electrophoretic mobility of in vitro produced and native proteins were compared on Western blot (Fig. 1B). Only one protein was revealed for *VvMSA-UB* and *VvMSA-CS*, with apparent molecular mass at about 24-kD and 23.4-kD, respectively. The both VvMSA proteins were detected in CS, corresponding to the full length and the truncated forms, and only one full size VvMSA protein in the UB cultivar.

3.2. Are the two VvMSA nuclear proteins?

Previously, we demonstrated that the VvMSA-UB nuclear localization signal (NLS) is a functional one, required for the VvMSA targeting to the nucleus [9]. We isolated and purified nuclei from a CSB cell suspension and checked their integrity by staining with the Hoechst reagent and propidium iodide (Fig. 2A and B). Nuclear proteins were separated into two different fractions. The first one carried mainly nucleosolic proteins, and the possible contaminations of cytosolic proteins (Fig. 2C). The second corresponded to histone proteins and some acid soluble, non-histone proteins. The identification of this nuclear fraction was confirmed by immunodetection of grape histone H1 proteins (Fig. 2C) with a specific antibody raised against histones H1 from pea [15]. Analysis by Western blotting demonstrated the presence of both, full length and truncated, VvMSA proteins in this second fraction containing histones and acid-soluble non-histone chromosomal proteins.

3.3. Searching for VvMSA protein partners

To identify grape ASR interacting proteins, the yeast two-hybrid approach was applied, using VvMSA-UB protein as bait. The expression of the fusion protein BD-GAL4/VvMSA-UB in the yeast host strain was immunodetected with the specific anti-VvMSA antibody (Fig. 3A). After transformation of this yeast reporter strain with the prey cDNA library, from grape berry, primary genetic screenings revealed several putative clones. To discriminate between spontaneous yeast

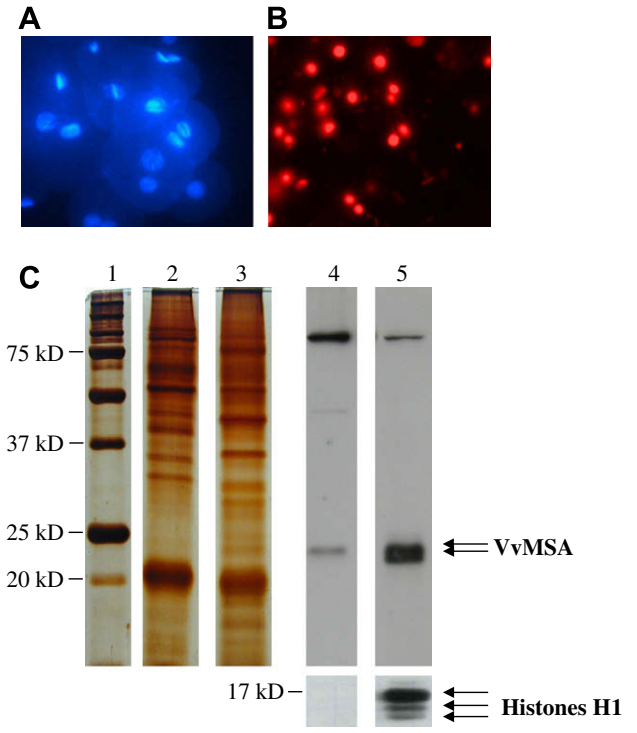

Fig. 2. Fractionation of nuclear proteins. (A) Hoechst-33258 staining of grape cells (magnification ×40). (B) Propidium iodide staining of isolated nuclei (magnification ×100). (C) Protein separation by SDS–PAGE (16%): 1. Protein markers; 2 and 4. Fraction of nucleosolic proteins; 3 and 5. Fraction of acid-soluble chromosomal proteins. Lanes 2 and 3. Silver staining; 4 and 5. Immunodetection with anti-VvMSA and anti-pea Histone 1 antibodies (1/1000).

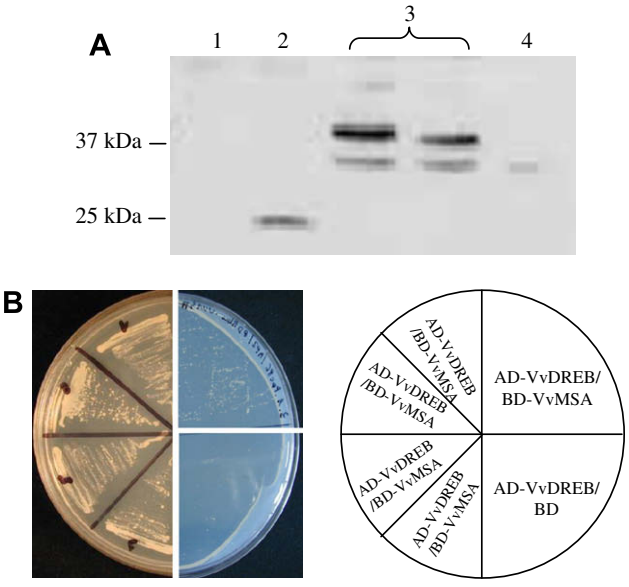

Fig. 3. Two-hybrid screening for VvMSA partner. (A) Immunodetection of Gal4-BD/VvMSA-UB fusion protein: 1. Protein markers; 2. TnT-produced VvMSA-UB (positive control); 3. Fusion protein expressed in yeast; 4. Proteins from untransformed yeasts (negative control). (B) Growth of a positive clone (four different colonies) on Ura-depleted medium (left half); Verification of VvMSA/VvDREB interaction (upper right panel) and VvDREB auto-activation of growth (lower right panel).

mutations and true positive clones, we retransformed the yeast reporter strain with the cDNA of each potential candidate. Finally, two positives clones encoding the same transcription factor were isolated (Fig. 3B). As a control, the absence of activation of yeast reporter genes by this putative partner was verified.

3.4. Sequence analysis of the VvMSA protein partner

The isolated cDNA is 722 bp long, encompassing an ORF of 459 pb. The predicted protein has 152 amino acids, pI – 8.67 and M_r – 17 kD (Fig. 4A). This protein displays the highest homology with DREB transcription factors [16], and was named VvDREB for *Vitis vinifera* Drought Response Element Binding protein.

VvDREB contains an APETALA2 (AP2) domain of 60 amino acid residues at its N-terminal region, from position 23 to position 82, which shares 98.3% of similarity with plant-specific AP2 domains (Fig. 4A) and encompasses two specific sequence blocks. The first consists of about twenty amino acids carrying the YKG (YRG) conserved motif, required for binding to DNA. The second one is at about 40 amino acids and displays the conserved sequence RAYD, predicted to form an amphipathic α-helix for protein–protein interaction [17]. However, VvDREB does not display any DREB1/CBF specific signatures [18] and its place in the phylogenic tree (Fig. 4B) indicates that it belongs to the DREB2 subfamily, mostly involved in dehydration and osmotic stress, rather than in cold responses [16,19].

3.5. Study of VvMSA and VvDREB interaction

To investigate the interaction of both partners in plant cells, we used the BiFC approach [20]. In order to apply this method

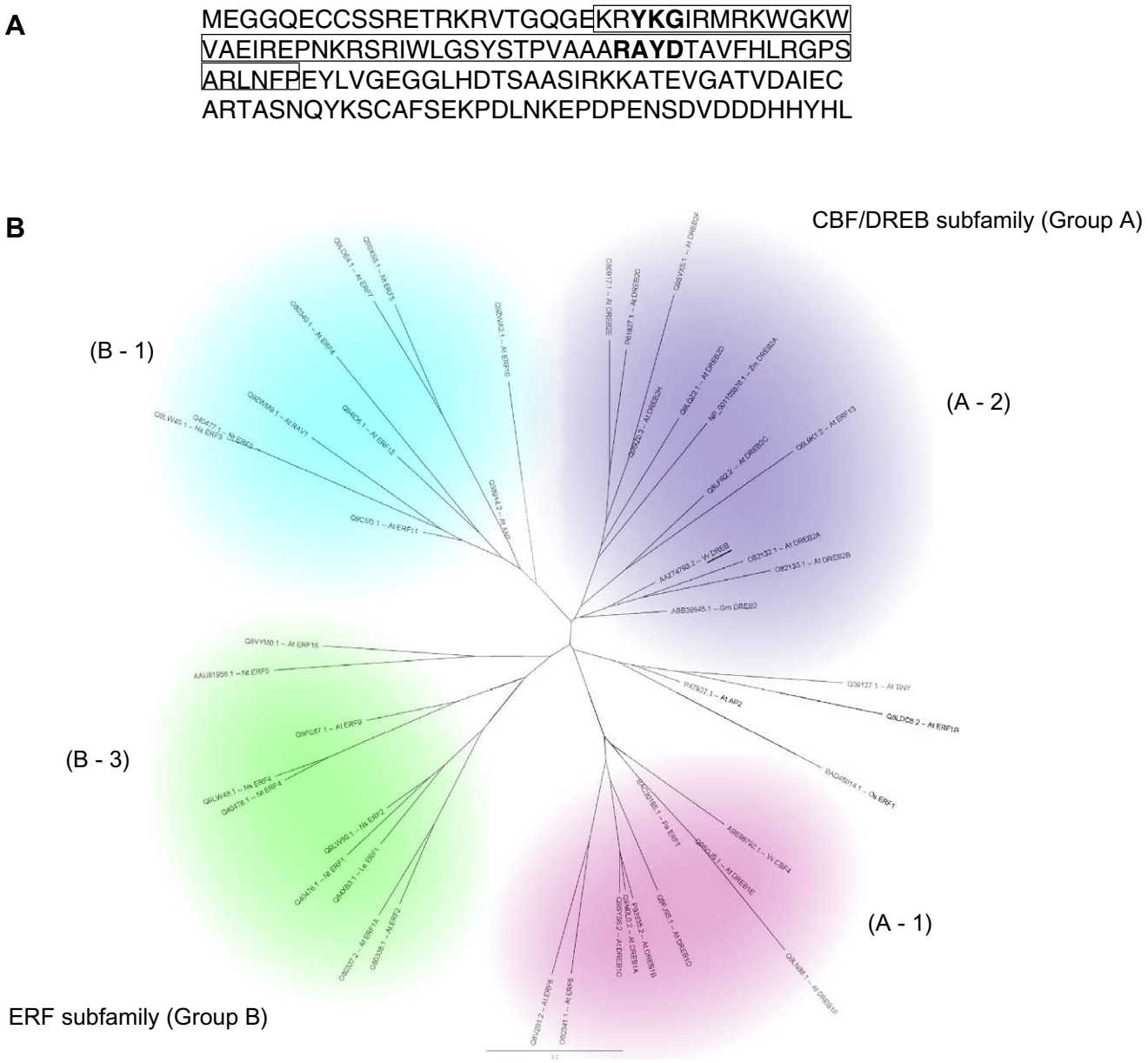

Fig. 4. Sequence analysis of the VvDREB protein. (A) Amino acid sequence and characteristic elements: AP2/ERF domain is boxed; YKG and RAYD motifs are underlined. (B) Phylogenetic tree of AP2/ERF proteins (CLUSTAL W, Neighbor-Joining).

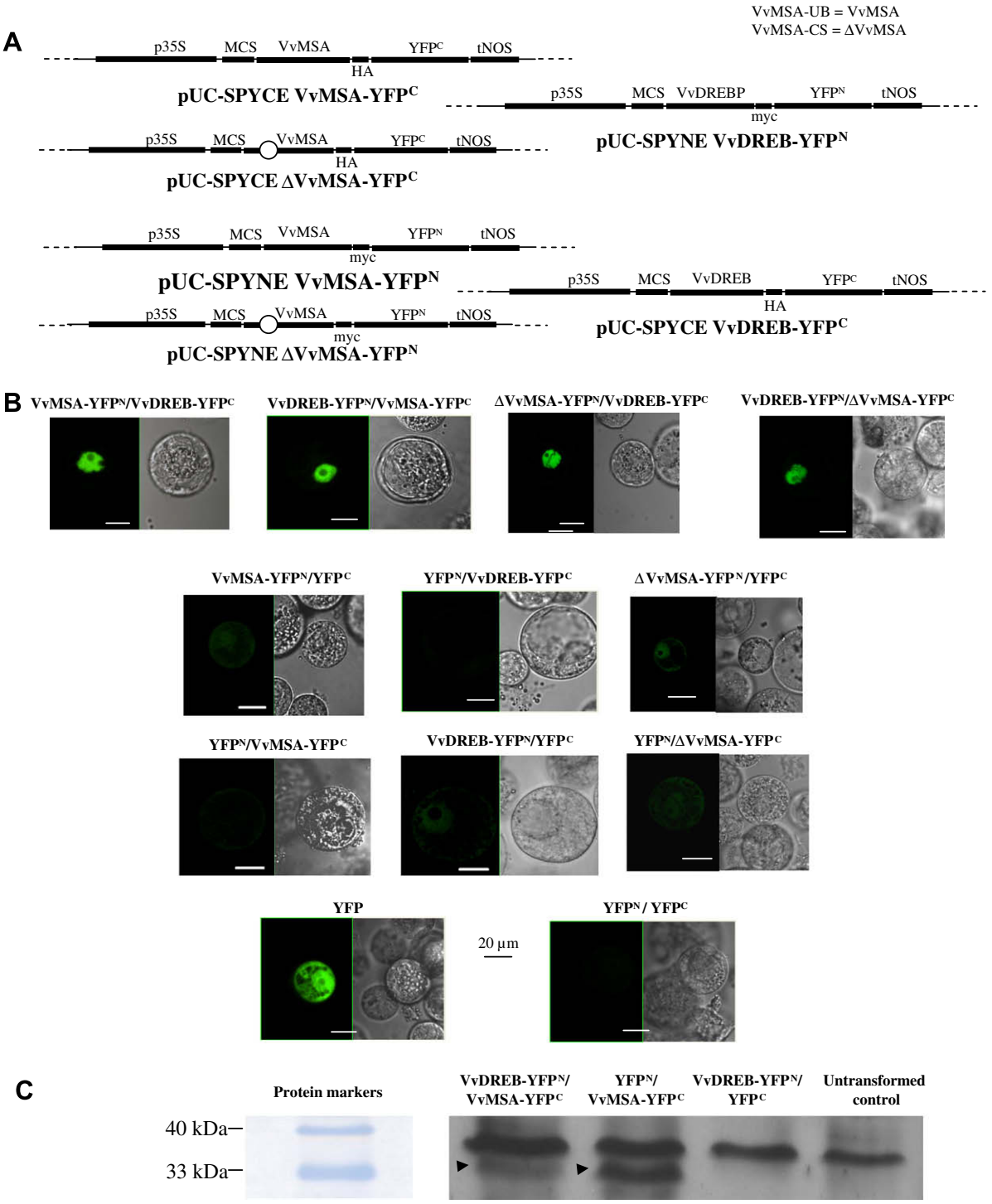

Fig. 5. Interaction of VvMSA proteins with VvDREB. (A) Fusion protein constructs. (B) Confocal microscopy visualization: VvMSA/VvDREB interactions (upper panels); specific controls (middle panels); positive control (lower left panel) and negative control (lower right panel). (C) Immunodetection of fusion protein VvMSA-YFP^C in grape protoplasts with anti-VvMSA antibody. A total protein extract from 150 μL suspension containing 2×10^5 protoplasts/mL is loaded by well.

to the homologous system, we developed a protocol for transient expression in protoplasts isolated from grape suspension cultured cells CSB. The constructs were obtained in pUC-SPYNE and pUC-SPYCE vectors, for split YFP N-terminal and YFP C-terminal fragment expression, respectively [21]. Both partners, VvMSA-UB and VvDREB were produced as fusion proteins with each of the two respective parts of YFP (Fig. 5A).

As visualized in Fig. 5B, VvMSA and VvDREB interaction in both protein fusion combinations is confirmed by a strong fluorescence exclusively concentrated in the nucleus. This spectacular confinement of fluorescence in the nuclear compart-

ment due to the interaction of protein partners was in contrast to the fluorescence observed in both cytoplasm and nucleus of the positive control (the complete YFP), and the almost total absence of fluorescence in the negative control (empty plasmids pUC-SPYNE and pUC-SPYCE). In specific controls, displaying each partner fusion against the complementary empty vector, a background level of fluorescence was detectable, but always in both compartments.

The presence of VvMSA-UB as fusion protein after transient expression was checked by immunodetection with anti-VvMSA antibody (Fig. 5C). The VvMSA-YFP^C protein (33.8 kD) was observed in transformed protoplasts and was lacking in untransformed controls.

3.6. Is the truncated version of the VvMSA protein able to interact with VvDREB?

To answer this question, two other constructs were produced corresponding to the fusions of truncated VvMSA protein with YFP^N or YFP^C parts (Fig. 5A), and used to study the interaction with VvDREB by the BiFC approach. Observation by confocal microscopy unambiguously demonstrated the efficient interaction of both protein partners, truncated VvMSA and VvDREB (Fig. 5B). Furthermore, the interaction of these transcription factors was exclusively confined in the nucleus, thus providing the evidence that the two grape ASR forms, full length and truncated version, correspond to functional proteins with respect to the studied protein–protein interaction.

4. Discussion

Cloning and in-depth analysis of grape ASR in both varieties "Cabernet-Sauvignon" and "Ugni blanc" revealed the existence of a polymorphism due to a five amino-acids deletion in VvMSA protein. Furthermore, the analysis in silico of eight tentative consensuses corresponding to 1353 EST 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, only of the full-length form in Ugni blanc, and only of the truncated protein in Pinot noir, the latter confirmed by the Pinot noir genome analysis [22]. Thus, our experimental results and in silico data for VvMSA proteins in Ugni blanc and Cabernet-Sauvignon strongly suggest that grape ASR proteins may be represented by one of the forms, full length or truncated, or by both of them.

A crucial point in the present work is the identification of VvDREB as a partner of the VvMSA proteins (Fig. 3B). The direct interaction of VvMSA and VvDREB is clearly visualized by the BiFC approach in grape cells. The confinement of fluorescent signal in the nucleus unambiguously suggests that the interaction of both transcription factors is involved in gene expression regulation (Fig. 5B). The demonstration that both, full length and truncated, grape ASRs interact with VvDREB exclusively in the nucleus highlights the physiological importance of VvMSA proteins (Fig. 5B). These findings and the in silico analysis imply that the two VvMSA proteins are functionally active.

Up to now, homodimer formation has been reported for tomato ASR1 by atomic force microscopy [23] and chemical cross-linking experiments [24]. Our results provide evidence that grape ASR are able to form a hetero-protein complex interacting with another transcription factor, VvDREB.

Another major point of this work is that grape ASRs are unambiguously identified as chromosomal non-histone proteins (Fig. 5C). The affiliation of ASRs to the chromosomal non-histone proteins, their capacity of binding to DNA [13,9,14,25,26], their involvement in protein–protein interactions [23,24, present work] and the relative abundance of these ASR proteins at different stages of development and in response to environmental cues [2,9,10] indicate a possible role as transcription factors of architectural type.

In conclusion, we demonstrate that grape ASRs are chromosomal non-histone proteins, which might be transcription factors of architectural type, which may function through recruitment of other transcription factors such as VvDREB.

Note

The accession numbers for VvMSA and VvDREB are AF281656 and DQ097182, respectively.

Acknowledgements: We are grateful to Dr. Klaus Harter (University of Köln, Germany) for gift of pUC-SPYNE and pUC-SPYCE vectors, Dr. Marinus Pilon (Colorado State University) for critical reading of the manuscript, Dr. Pierrette Fleurat-Lessard, Dr. Matthieu Régnacq and Dr. Rémi Lemoine (University of Poitiers). This work was supported by la Région Poitou-Charentes and le Ministère de l'Enseignement Supérieur et de la Recherche.

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