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Characterization, functional validation and gene expression patterns of two 14-3-3 isoforms from *Vitis vinifera*

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Summary

In eukaryotic cells, 14-3-3 proteins constitute a family of conserved regulatory proteins which bind to a large number of phosphorylated partners. Although a great body of evidences accumulated in herbaceous species indicates that 14-3-3s play key roles in regulation of plant growth, information about function in tree plants is still very scarce. In this paper, we report the characterization of two novel 14-3-3s from grapevine (*Vitis vinifera* 'Cabernet Sauvignon') named *VV1_CS* and *VV2_CS*. The *VV1_CS* and *VV2_CS* cDNA were expressed in *Escherichia coli* and the recombinant proteins were shown to be functional proteins using plasma membrane H^+-ATPase as target. Transcript analysis during grape development or under elicitor-induced stress revealed differential expression of the two 14-3-3 isoforms.

Key words: 14-3-3 proteins, *Vitis vinifera*.

Abbreviations: AHA1, Arabidopsis H^+-ATPase isoform 1; FC, fusicoccin; VV1_CS, *Vitis vinifera* 14-3-3 isoform 1 'Cabernet-Sauvignon'; VV2_CS, *Vitis vinifera* 14-3-3 isoform 2 'Cabernet-Sauvignon'.

Introduction

14-3-3 proteins are a family of conserved dimeric proteins involved in the regulation of a wide range of many cellular processes in eukaryotes. Dimerization of 14-3-3 monomers into homo- or heterodimers allows these proteins to interact with many diverse targets through binding to conserved consensus motifs (Yaffe 2002). Plants have a variable number of 14-3-3 isoforms: *Arabidopsis* contains 13 isoforms (DeLille *et al*. 2001), cotton six (Zhang *et al.* 2010), rice eight (Yao *et al.* 2007) and barley five (Schoonheim *et al.* 2007); soybean contains sixteen transcripts (Li and Dhaubhadel 2011), while tobacco has 17 potential isoforms (Konagaya *et al.* 2004). The Grape Genome Browser (http://www.genoscope.cns.fr/externe/GenomeBrowser/Vitis/), created by a French-Italian Public Consortium for Grapevine Genome Characterization, identifies to date not less than seven putative 14-3-3 isoforms (Jaillon *et al.* 2007). In agreement with their common origin, there is a high sequence conservation within and among species (Wang and Shakes 1996). Within a species, 14-3-3s are divided into multiple groups or clades that may have different physiological roles (Li and Dhaubhadel 2011, Piotrowski and Oecking 1998).

In plants, 14-3-3 proteins are involved in several fundamental physiological processes, such as primary metabolism, ion transport, cell division and growth, hormonal signalling, as well as environmental adaptation, including response to light or to biotic and abiotic stresses (Denison *et al.* 2011, Aducci *et al.* 2002). The role of 14-3-3s in the control of cell energy is also indicated by the fact that they can bind AMP, which prevents their interaction with other partners such as H^+-ATPase (Camoni *et al.* 2001), the master enzyme of ion transport across the plasma membrane (Michelet and Boutry 1995). 14-3-3 isoforms exhibit tissue-specific expression patterns during barley embryo development (Fulgosi *et al*. 2002). At the systemic level, it is known that 14-3-3 proteins are involved in regulation of hormone pathways. 14-3-3s have well-known roles in brassinosteroid (Gampala *et al.* 2007, Kim *et al.* 2009) and abscisic acid signaling (Cutler *et al.* 2010, Schoonheim *et al.* 2007, del Viso *et al.* 2007, Hong *et al.* 2011) as well as in gibberellic acid biosynthesis (Schwechheimer and Willige 2009, Ishida *et al.* 2004). A growing body of evidences also pinpoints a relevant role of 14-3-3 proteins in response of plants to abiotic and biotic stresses (Yang *et al.* 2009). Environmental stresses can regulate the expression and phosphorylation of 14-3-3s (Sun *et al.* 2011).

Most of the published results about the roles of plant 14-3-3 have been obtained in herbaceous/model species whereas data in tree/crop species are still very scarce, although a deeper understanding of 14-3-3 role in metabolic or developmental processes of crop species, such as for instance fruit development and ripening, could be very helpful for improvement of growth and productivity. Grapevine (*Vitis vinifera*) is one of the world's most widespread fruit crop, and during the last years, due to its economical relevance, it has become a model species for ripening studies. The present study describes the identification and characterization of two novel 14-3-3 isoforms from grape berries. Their expression profile in grape berries during development/ripening, as well as in leaves in response to elicitor challenge, has been analyzed.

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Dedicated to the memory of Prof. Saïd Hamdi (1957-2006).

Material and Methods

Elaboration of the 14-3-3 phylogenetic tree: Database searches were conducted with the basic local alignments search tool of the National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and *via* the DFCI Grape Gene Index (http://compbio.dfci.harvard.edu/cgi-bin/tgi/gimain.pl?gudb=grape). Alignment of amino acid sequences was performed using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) with default parameters. Phylogenetic tree (using the maximum parsimony method with 1000 bootstrap replications) were made by the software MEGA5 (http://www.megasoftware.net/). Phylogenetic tree was performed by aligning 14-3-3 sequences from *Vitis vinifera* (VV1_CS, GenBank accession number ACO40495; VV2_CS, GenBank accession number ACO40494) and from *Arabidopsis thaliana*, accession numbers: At κ (AAD51783), At λ (AAD51781), At χ (AAA96254), At φ (AAB62224), At ω (AAA96253), At ν (AAD51782), At υ (AAB62225), At ψ (AAA96252), At μ (AAD51784), At ι (AAK11271), At ο (AAG47840), At ε (AAD51785), At π (NP_565174). The *Entamoeba histolytica* HMI-IMSS 14-3-3-1 isoform (Eh14-3a), accession number: U13418 was used as out-group because of its distance from the other 14-3-3s (Piotrowski and Oecking 1998). For the analysis of the C-terminal end of grapevine 14-3-3s the PHD software was used (Rost and Sander 1993).

Chemicals and reagents: Oligonucleotides were obtained from Eurogentec (Belgium). Glutathione Sepharose 4B from GE Healthcare (Uppsala, Sweden). Other chemicals were of analytical grade and were purchased from Sigma (St. Louis, Missouri, USA). Restriction enzymes were from Roche Diagnostics (Mannheim, Germany). Pfu turbo DNA polymerase was from Stratagene (La Jolla, California, USA). Protein standards (Precision plus protein standards) and other chemical for SDS-PAGE were from Bio-Rad (Hercules, California, USA).

Vitis 14-3-3 cDNA cloning and sequence analysis: A cDNA library of grape berries collected at ripening stage from 'Cabernet Sauvignon' variety has been constructed using Smart cDNA cloning kit Clontech (USA). ESTs cloned into *pTriplEx* were sequenced (Genome Express, France) and referenced by the DFCI Grape Gene Index.

Both *VV1_CS* and *VV2_CS* cDNA were amplified from *pTriplEx* by PCR using *Pfu* (Promega) and specific primers for the *VV1_CS* gene (forward primer 5'-AATGGATCCATGGCGGCAGCTCCATCC-3', reverse primer 5'-AATGGATCCTCACTGCTGTTCATCATCAC-3') and for the *VV2_CS* gene (forward primer 5'-CGGCAGCTCCATCCGCACGC-3', reverse primer 5'-GCCGAGGGAATYCAGCCGGG-3').

The *VV1_CS* and *VV2_CS* amplicons were subcloned into pGEM-T easy vector (Promega) and sequenced. These constructs were digested with appropriate enzymes and cloned into pGEX-2TK (GE Healthcare) at BamHI site for *VV1_CS* or between BamHI and EcoRI sites for *VV2_CS*. The authenticity of sequence constructs was confirmed by restriction analysis and DNA sequencing (Genome express, France). Recombinant plasmids were electroporated in *E. coli* M15 strain (Stratagene, La Jolla, California, USA).

Recombinant protein expression and purification: VV1_CS and VV2_CS 14-3-3 proteins were expressed in M15 *E. coli* as fusion proteins with the glutathione S-transferase (GST), using pGEX-2TK vector and purified by affinity chromatography using Glutathione-Sepharose 4B (Fullone *et al.* 1998).

SDS-PAGE and immunoblotting: SDS-PAGE (Laemmli 1970) was performed in a Mini Protean apparatus (Bio-Rad). Thrombin cleavage of the fusion protein GST-14-3-3 was performed when GST-fusion proteins were immobilized on the glutathione-sepharose beads in order to remove GST from the samples. For immunoblotting, samples of affinity-purified GST-14-3-3 (3 μg) or crude protein extract from grape berries (25 μg) were subjected to SDS-PAGE and blotted onto nitrocellulose membrane, using a semidry LKB apparatus (2 h, 0.8 mA cm^{-2}). Blots were probed with anti-14-3-3 antibodies (1/500) (Fullone *et al.* 1998). Polypeptides were visualized by alkaline phosphatase-conjugated secondary antibodies (1/10000) (Sigma), using enhanced chemiluminescence detection (ECL kit, GE Healthcare).

Overlay assay (Far Western Blotting): pGEX-2TK expression vector produces a GST-fused protein containing a cAMP-dependent protein kinase phosphorylation site and a thrombin site between the two polypeptides. The ^{32}P-labeled VV1_CS and VV2_CS proteins were obtained as already described (Fullone *et al.* 1998). Briefly, 200 μL of *E. coli* lysate containing GST-fused VV1_CS and VV2_CS proteins were incubated with 35 μL of glutathione-sepharose beads. After washing with phosphate-buffered saline (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 1.8 mM KH_2PO_4, pH 7.3), beads were incubated in 30 μL of buffer A (20 mM Tris-HCl, 12 mM $MgCl_2$, 100 mM NaCl, pH 7.5) with 1.11 MBq of [γ-^{32}P]ATP (110 TBq·$mmol^{-1}$) and 10 units of the catalytic subunit of protein kinase A (Sigma) for 30 min at 30 °C. The beads were washed three times with 1 mL of phosphate-buffered saline and then incubated in 100 μL of phosphate-buffered saline with 1 unit of thrombin (GE Healthcare) overnight at 22 °C. The mixture was centrifuged and the supernatant was collected. ^{32}P incorporation was measured in a liquid scintillation β-counter (LKB Wallac 1410). Specific activities of both labelled proteins were similar (about 90 GBq/mmol). The overlay assay was carried out as already described (Fullone *et al.* 1998), with minor modifications. Briefly, two-phase partitioned maize plasma membranes (10 μg protein) containing the H^+-ATPase were subjected to SDS-PAGE and blotted onto nitrocellulose membrane, using a semidry LKB apparatus (2 h, 0.8 mA cm^{-2}). The membrane was blocked with 5 % fat-free milk in 25 mM Hepes-OH, 75 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 0.1 mM EDTA, 0.05 % Tween-20, pH 7.5 (buffer H) and then incubated overnight at 4 °C in the same buffer, containing 2 % fat-free milk, 0.1 nmol of ^{32}P-labeled VV1_CS or VV2_CS (corresponding to 9 kBq·mL^{-1}). After incubation, the membrane was washed three times

with buffer H, dried and subjected to autoradiography at -80 °C. Each overlay experiment was performed at least three times and similar results were obtained.

Purification of endoplasmic reticulum from yeast expressing AHA1: Plasma membrane H^+-ATPase AHA1 isoform was expressed in *Saccharomyces cerevisiae* as previously described (Regenberg *et al.* 1995). After cell homogenization, membranes were purified by differential centrifugation and endoplasmic reticulum, containing most of the AHA1, was isolated by sucrose gradient centrifugation (Marra *et al.* 2000).

Phosphohydrolytic activity assay: AHA1 phosphohydrolytic activity of yeast endoplasmic reticulum (ER) membranes was assayed as already described (Marra *et al.* 2000, Camoni *et al.* 2012) with minor modifications: 10 µg of sucrose gradient purified yeast ER were preincubated with 10 µg of VV1_CS or VV2_CS protein in 500 µL of 50 mM Tris-Mes, 5 mM $MgCl_2$, 50 mM KNO_3, 5 mM NaN_3, 0.2 mM ammonium molybdate, pH 7.2 containing 10 µM fusicoccin. After 20 min incubation, 2 mM ATP was added. ATP hydrolysis was measured as already described (Serrano 1989).

Plant growth conditions: Rooted cuttings of *Vitis vinifera* L. 'Cabernet Sauvignon' were grown in a greenhouse (maximum temperature 24 °C) in potting soil irrigated with water. Leaves, stems and roots were collected from 2-month-old rooted plants and frozen in liquid nitrogen until used.

Elicitation was made by spraying 1 mL of solution on the leaves containing known quantities of the molecule. Elicitors used for analysis were: jasmonic acid (150 µM in 1 % ethanol), ethephon (10 mM in water), salicylic acid (5 mM in water), H_2O_2 (10 mM in water), and ergosterol (200 µM in 2 % ethanol, 0.02 % triton) (Marchive *et al.* 2007). For each time point of the kinetics, leaves from three plants were collected, combined and dipped in liquid nitrogen and stored at -80 °C until used for RNA extraction. Berries from *Vitis vinifera* L. cultivar 'Cabernet Sauvignon' were sampled during the 2004 growing season. Each week, a sample of fifty randomly selected berries from three bunches was harvested. Stages of berry development were determined according to usual criteria (size, measures of soluble solids, softening and berry colour) as previously described (Boss *et al.* 1996). For the identification of developmental stages, the "modified Eichorn-Lorenz system" (modified E-L system) was used (Coombe 1995). Berries were frozen in liquid nitrogen and stored at -80 °C until use.

RNA extraction and RT-PCR experiments: Total RNA was extracted from three independent samples of leaves, stems, roots and berries at different stages of development (Reid *et al.* 2006). First strand cDNA synthesis was performed using 2 µg of total RNA, oligo(dT) as primer and M-MLV reverse transcriptase (Promega, Madison, WI, USA). PCR amplification using cDNAs as template was performed with gene-specific primer pairs listed below. *VV1_CS* gene: forward primer 5'-AATGGATCCATGGCGTCCACACTGCCG-3', reverse primer 5'-AATGAATTCCTATGGCTCATCCAACTG-3'. *VV2_CS* gene: forward primer 5'-GCGTCCACACTGCCGGAG-3', reverse primer 5'-AAATACCAGCACAGACCTCG-3'. An initial denaturation step (5 min at 95 °C) was followed by 25 to 35 cycles of 5 s at 95 °C, 45 s at 55 °C, and 1 min at 72 °C. PCR fragments were run on a 1.5 % (w/v) agarose gel containing ethidium bromide and visualized by UV. Fragments were purified and sequenced for identification (Genome Express, France). The expected amplicon sizes were 400 bp and 420 bp for *VV1_CS* an *VV2_CS*, respectively. The amplification of the elongation factor *VV-EF1-γ* cDNA (forward primer 5'-CAAGAGAAACCATCCCTAGCTG-3', reverse primer 5'-TCAATCTGTCTAGGAAAGGAAG-3') was used as an internal and normal control which has a constant profile over time (Marchive *et al.* 2007). Each ethidium bromide-stained band on the gel was quantified using Quantity One software from Bio-Rad (Lima *et al.* 2012).

Statistical analysis: Two independent experiments with three technical replicates were performed for each assay, while only one representative experiment is shown. Error bars were produced by calculating the standard deviation. The gene expression analysis was performed on two independent experiments.

Results

Characterization and in silico analysis of *Vitis vinifera* cDNA clones VV1_CS and VV2_CS: Two 14-3-3 full-length cDNA were identified in the INRA EST bank. One cDNA, called VV1_CS, contained a 1157 bp insert encoding a 260 residue polypeptide with a calculated molecular weight of 29.42 kDa. The second one, called VV2_CS, contained a 1036 bp insert encoding a 252 residue polypeptide with a calculated molecular weight of 28.65 kDa. The VV1_CS and VV2_CS proteins showed 78.5 % identity at the amino-acid level and 67.9 % identity at nucleotide sequence level (data not shown). The two protein sequences were compared with a typical 14-3-3 of *Arabidopsis*, the 14-3-3 epsilon, and with the Arabidopsis 14-3-3 omega (Fig. 1). The alpha helices contained in all the 14-3-3s, as well as the five blocks of conserved sequences, are shown (Wang and Shakes 1996, Ferl *et al.* 1994). Two 14-3-3 signatory motifs are present in the two isoforms from grape berry (Wang and Shakes 1996): RNLLSVAYKNVI in block 2 and SYKDSTLIMQLLRDNLTWTSD in block 5, both identical in the two isoforms.

A phylogenetic tree comparing the two grapevine 14-3-3 isoforms with the *Arabidopsis* 14-3-3s (Fig. 1b) was made using the method of maximum parsimony. Group identification was performed according to the work of Piotrowski and Oecking (1998) and an "out-group", represented by an *Entamoeba histolytica* 14-3-3 was added (Piotrowski and Oecking 1998). The results show that VV1_CS and VV2_CS belong to the non-epsilon group of 14-3-3 proteins previously described (DeLille *et al.* 2001) but they fall in two distinct groups, the Omega-group and the Kappa-group respectively for VV1_CS and VV2_CS, according to Pio-

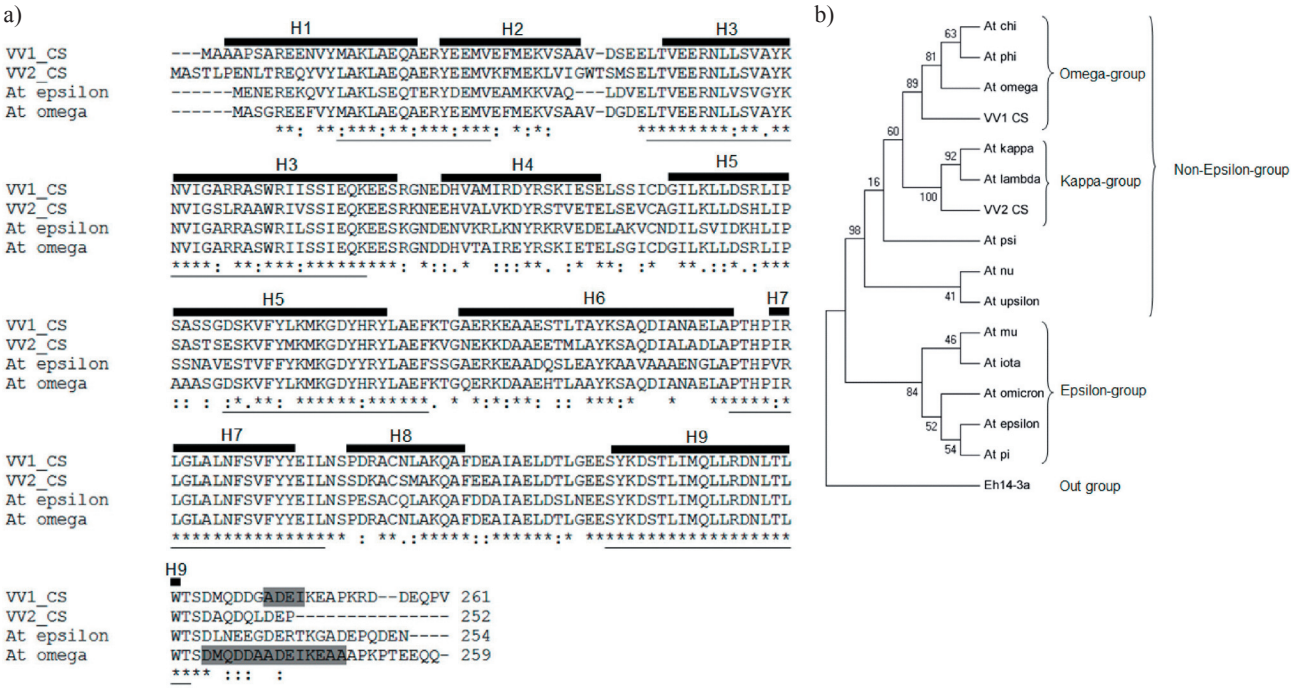

Fig. 1: Comparison of amino acid sequences of 14-3-3 proteins. **a**): Sequence alignment of *V. vinifera* 14-3-3 proteins, the epsilon and omega isoform from *Arabidopsis thaliana*. Multiple sequence alignment was performed with the Clustal Omega software. Black bars indicate the position of the nine α-helices. Underlines indicate the five conserved sequences in all 14-3-3s. In grey are indicated the theoretical tenth alpha-helix. **b**): Phylogenetic tree of 14-3-3 proteins from *Arabidopsis thaliana* (DELILLE *et al.* 2001) and *Vitis vinifera* constructed with the MEGA5 software using the maximum parsimony method. The 14-3-3 named "Eh14-3a" is the out-group from *Entamoeba histolytica*.

TROWSKI and OECKING (1997). A further analysis was done comparing the C-terminals from VV1_CS and VV2_CS and the *Arabidopsis* 14-3-3 omega isoform, which has been proposed to contain a tenth alpha helix (SHEN *et al.* 2003). The *in silico* analysis does not show any theoretical alpha-helix in the VV2_CS C-terminal region (Fig. 1a), whereas four residues in the VV1_CS C-terminal are compatible with an alpha-helix structure, similarly to what observed for the Arabidopsis 14-3-3 omega (SHEN *et al.* 2003).

Characterization of VV1_CS and VV2_CS proteins expressed in *E. coli*: *V. vinifera* 14-3-3 proteins were expressed in *E. coli* as GST (Glutathione S-Transferase)-fusion proteins and purified by affinity chromatography. The SDS-PAGE of purified proteins is shown in Fig. 2. Lanes 1 and 3 show the purified VV1_CS and VV2_CS proteins as GST-fusion

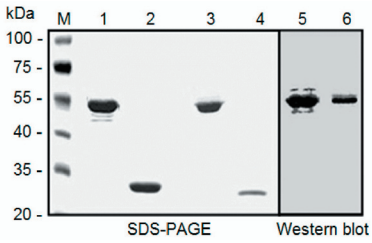

Fig. 2: SDS-PAGE and immunoblotting of VV1_CS and VV2_CS expressed in *E. coli* as GST-fusion proteins. Left panel (lanes 1 to 4): coomassie-stained gel. Right panel (lanes 5 and 6): immunoblotting of GST-fused VV1_CS and VV2_CS with anti-14-3-3 antibodies. GST-fused (lanes 1, 3, 5 and 6) or thrombin-cleaved (lanes 2 and 4) VV1_CS (lanes 1, 2 and 5) and VV2_CS (lanes 3, 4 and 6) proteins purified by affinity chromatography were loaded on the gel.

proteins, while lanes 2 and 4 show VV1_CS and VV2_CS proteins after thrombin cleavage, to remove the GST protein. The apparent molecular weight was of about 30 kDa for both proteins, in accordance with the calculated molecular weights of VV1_CS (29.42 kDa) and VV2_CS (28.65 kDa) proteins. Lanes 5 and 6 show the Western blotting of both GST-fused 14-3-3 proteins. Anti-14-3-3 antibodies, raised against a conserved plant 14-3-3 sequence, were able to immunodecorate both the GST-fused VV1_CS and VV2_CS proteins.

In order to verify the functionality of the recombinant proteins, their ability to interact with the H^+-ATPase and to stimulate its activity was assayed. Interaction studies were performed by means of an "overlay assay" using the ^{32}P-labelled VV1_CS or VV2_CS proteins as probes and a crude plasma membrane preparation from maize roots containing the H^+-ATPase as a bait. The autoradiography (Fig. 3a) demonstrated that both 14-3-3 proteins were able to bind to the H^+-ATPase and that VV1_CS interacted to a higher extent, as compared to VV2_CS. The interaction was specific, since no binding with any other protein present in the crude plasma membrane fraction was observed.

To test the effect of 14-3-3 proteins on the H^+-ATPase activity, a recombinant AHA1 H^+-ATPase isoform (*Arabidopsis* H$^+$- ATPase isoform 1) expressed in yeast was used. In this system, most of the functional enzyme is located at the endoplasmic reticulum (ER) (VILLALBA *et al.* 1992). A significant and reproducible stimulation of the H^+-ATPase phosphohydrolytic activity can be induced by exogenous 14-3-3, providing that fusicoccin (FC) is added to the reaction mixture (CAMONI *et al.* 2001, VISCONTI *et al.* 2003). ER membranes were incubated with the GST-VV1_CS or

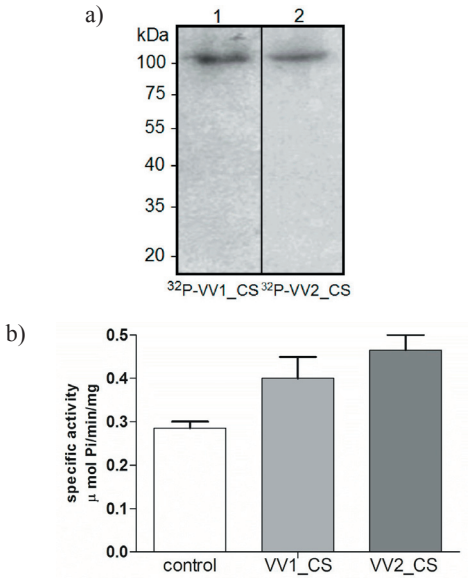

Fig. 3: Functional characterization of VV1_CS and VV2_CS. **a**): Association of 14-3-3 proteins with the H^+-ATPase. The overlay assay was performed with 10 µg of plasma membrane preparation from maize roots. **b**): Effect of 14-3-3 proteins on the phosphohydrolytic activity of AHA1 H^+-ATPase.

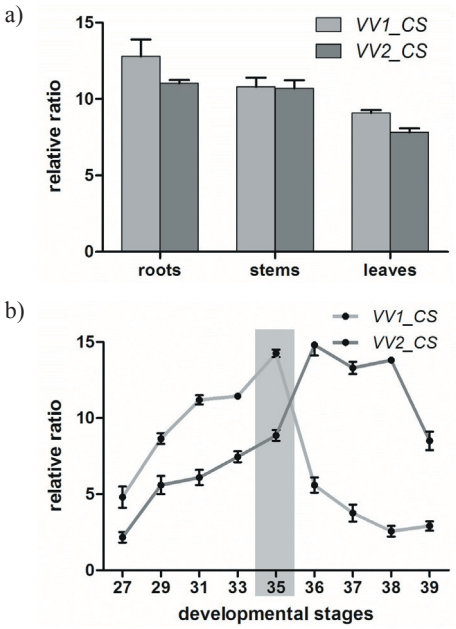

Fig. 4: Semiquantitative RT-PCR analysis of 14-3-3 gene expression in grape roots, stems, leaves and in berries. **a**): Roots, stems and leaves were collected from 2-month-old rooted plants. **b**): Grape berries at different stages of development (modified "E-L system" according to Coombe 1995). The grey bar (stage 35) represents the central stage of "véraison".

GST-VV2_CS fusion proteins in the presence of FC. The Fig. 3b shows that both VV1_CS and VV2_CS proteins were able to efficiently stimulate the enzyme activity and that VV2_CS brought about a higher stimulatory effect as compared to VV1_CS. Taken together, these results indicate that both recombinant 14-3-3 proteins from *V. vinifera* were able to associate with and to stimulate the H^+-ATPase, thus demonstrating that they were functionally active. Activities of both isoforms were comparable to values reported for other plant isoforms (Visconti *et al.* 2008).

VV1_CS and *VV2_CS* gene expression profiles: RT-PCR analysis was performed, in order to investigate the *VV1_CS* and *VV2_CS* gene expression profiles in berries at different developmental stages as well as in other grape organs.

Results demonstrated that *VV1_CS* and *VV2_CS*, besides berries, were expressed also in stems, roots and leaves (Fig. 4a). Expression studies performed with grape berries at different stages of development showed that the two transcripts were detected in all developmental stages but with different expression patterns (Fig. 4b). In fact, *VV1_CS* transcript increased constantly during the green stages (stages from 27 to 33) and at ripening (stage 35) and dramatically decreased during later maturation stages (stages from 36 to 39). *VV2_CS* transcript steadily increased during green stages, reaching a maximum at post-ripening (stage 36) and remaining constant until stage 38, to decrease only at the last maturation stage.

VV1_CS and *VV2_CS* expression in response to elicitors: To investigate the potential involvement of the two *Vitis* 14-3-3s in the response to stress, their transcript accumulation was evaluated after treatment of grape leaves with elicitors mimicking different biotic stresses. Jasmonic acid, ethephon (an ethylene-releasing compound), salicylic acid, hydrogen peroxide and ergosterol were supplied to grape leaves and *VV1_CS* and *VV2_CS* transcripts levels determined by semi-quantitative PCR analyses at different times, up to 24 h. As shown in Fig. 5, all elicitors were able to trigger accumulation of both 14-3-3 transcripts, although at different extent and with different time-courses. The highest level of transcript accumulation was brought about by ergosterol elicitation.

Discussion

14-3-3 proteins belong to a highly conserved protein family with regulatory roles in all eukaryotes, and peculiar functions in plants. 14-3-3 family is represented in different animal or plant species by a variable number of isoforms. *In silico* analysis separates isoforms into two main groups (the epsilon group and the non-epsilon group) and in more subgroups or clusters. It was proposed that the animal epsilon 14-3-3 is the most similar to plant 14-3-3s (Wang and Shakes 1996). This led to hypothesize that the epsilon isoform is a "living fossil" that has faithfully maintained the character of an ancestral 14-3-3 isoform (Wang and Shakes 1996, Doolittle 1994).

Both the two 14-3-3 isoforms identified from *Vitis* belong to the non-epsilon group, but they are sufficiently different to fall into two distinct clusters, omega and kappa respectively (Fig. 1b), their divergences being mostly located at the C-terminal regions. In particular, a part of the VV1_CS C-terminus is similar to that of *Arabidopsis* omega isoform, which has been proposed to contain a tenth alpha-helix (Shen *et al.* 2003).

In order to verify whether the two novel sequences represent functional proteins, the ability of recombinant isoforms to bind to and stimulate the activity of the plas-

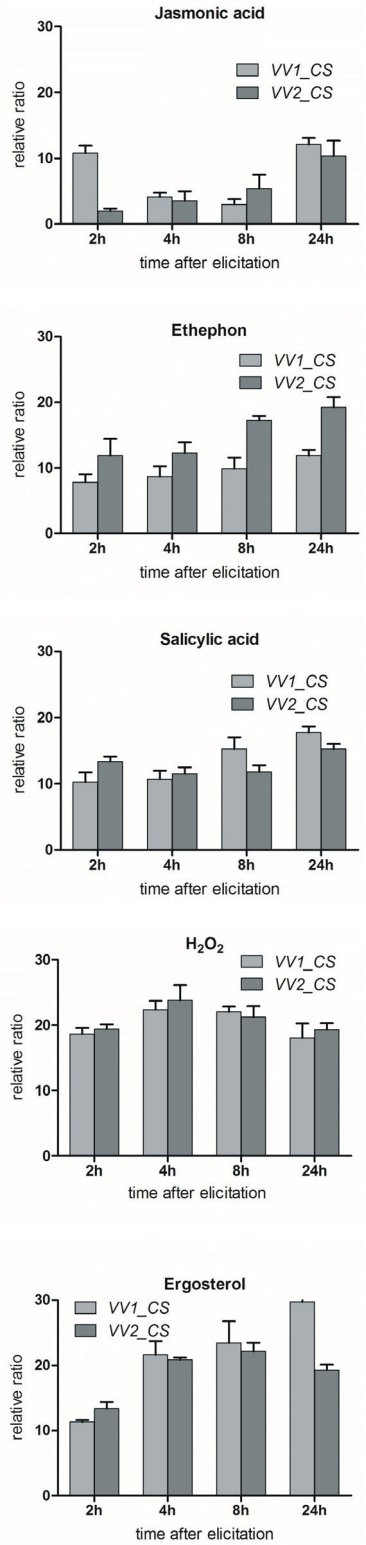

Fig. 5: Semiquantitative RT-PCR analysis of 14-3-3 gene expression in grapevine leaves treated with elicitors. Relative *VV1_CS* and *VV2_CS* levels were compared at 2, 4, 8 and 24 h to the inoculated control (0 h) and normalized to the reference gene EF1-γ.

ma membrane H^+-ATPase was assayed. *In vitro* produced 14-3-3s showed a size (about 30 kDa) comparable to that of known 14-3-3s, while activity assays showed that both *Vitis* isoforms were able to bind to and to stimulate the phosphohydrolytic activity of the plasma H^+-ATPase, thus demonstrating that the recombinant proteins are fully functional (Xu *et al.* 2012).

We also investigated the *VV1_CS* and *VV2_CS* expression in different grapevine tissues. Results indicated that *VV1_CS* and *VV2_CS* transcripts were present in roots, stems, leaves and berries, where they exhibited differential expression profiles during fruit development and ripening. In particular, analysis in berries clearly showed that *VV1_CS* and *VV2_CS* transcripts strongly increased at *véraison* (Fig. 4b), a crucial stage in the berry development, characterized by dramatic metabolic and anatomical changes (Coombe and McCarthy 2000, Zhang *et al.* 2008, Terrier *et al.* 2001). These results strongly suggest that these 14-3-3 isoforms play a relevant role in grapevine berry ripening and corroborate the hypothesis that 14-3-3 proteins may have a general role in the regulation of plant development (Daugherty *et al.* 1996, Li and Dhaubhadel 2011). 14-3-3 proteins are involved in plant responses to biotic stresses, such as powdery mildew infection (Yang *et al.*, 2009) or to abiotic stresses (Yao *et al.* 2007). In rice it was observed that 14-3-3s are elicited by both biotic and abiotic stresses (Chen *et al.* 2006). In the present work, compounds functioning as biotic stress elicitors have been used to investigate the potential involvement of the two novel isoforms in the response of *Vitis* leaves to biotic stress. Jasmonic acid and ethylene are molecules regulating the induced systemic resistance, which is associated to plant/bacteria interactions (Choudhary *et al.* 2007). Salicylic acid and hydrogen peroxide are molecules regulating the systemic acquired resistance (Hammerschmidt 2009), which is involved in the plant response to pathogens. Ergosterol, a molecule from fungal membranes, is perceived as a signal of pathogen invasion (Granado *et al.* 1995) and it has been proposed as a general elicitor in the pathogen associated molecular patterns (PAMPs)-triggered immunity (Rossard *et al.* 2010). All the tested elicitors were able to rapidly increase *Vitis* 14-3-3 transcript expression up to 24 h, thereby indicating that 14-3-3 proteins are involved in the early events of the plant defence response to pathogens (Jones and Dangl 2006). Accordingly, jasmonic acid affected transcription of 14-3-3 genes in rice (Chen *et al.* 2006), in hybrid poplar (Lapointe *et al.* 2001a) and in white spruce (Lapointe *et al.* 2001b). Jasmonate and ethephon induced the transcription of the 14-3-3 *Hb14-3-3c* in *Hevea brasiliensis* (Yang *et al.* 2012); salicylic acid caused an increase of 14-3-3 expression in potato (Aksamit *et al.* 2005); H_2O_2 induced 14-3-3 gene expression in rice (Chen *et al.* 2006, Yao *et al.* 2007) while in tomato the transcription of TFT6 14-3-3 isoform coincided with peaks of ethylene and salicylic acid production (Roberts *et al.* 2002). As far as ergosterol, to our knowledge, this is the first evidence of 14-3-3 transcript elicitation by this compound.

Conclusions

In conclusion, this study provides the identification and characterization of two 14-3-3 proteins from *Vitis vinifera*. Information about 14-3-3 from tree species is lacking and grapevine is a worldwide economically relevant tree cultivated species. Results allowed to ascertain that the two 14-3-3 isoforms are fully functional proteins, expressed in

different organs and that in berries they exhibit a differential pattern of expression during development and ripening. Furthermore, it has been demonstrated that in leaves 14-3-3 gene transcription is elicited by different compounds mimicking biotic stresses. These results suggest that also in grapevine 14-3-3s accomplish fundamental functions, both in plant development and adaptation, thereby prompting further studies aimed to a more detailed characterization of 14-3-3 expression profiles and biochemical functions in *Vitis vinifera*, particularly in relation to berry ripening and quality.

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