

14-3-3 proteins from *Vitis vinifera* bind to and modulate the anthocyanidin synthase activity *in vitro*

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Summary

14-3-3 proteins are regulatory proteins that play a major role in a lot of important processes in plants, but their role in the regulation of the secondary metabolism is still poorly documented. For the first time, we demonstrated that the activity of ANS, an important enzyme of the flavonoid pathway, can be modulated *in vitro* by 14-3-3 proteins in grapevine.

Key words: 14-3-3 proteins; anthocyanidin synthase; flavonoids; leucoanthocyanidin dioxygenase; *Vitis vinifera*.

Introduction

The 14-3-3s are a large family of regulatory proteins present in all eukaryotes. In plants, 14-3-3s have important roles in the response to stress, light, in primary metabolism, in the hormone signalling pathways and development (DENISON *et al.* 2011, WILSON *et al.* 2016), in defence and in plant-pathogen interactions (LOZANO-DURAN *et al.* 2015). They belong to a multigenic family and are expressed as several isoforms in most plants. The *Arabidopsis thaliana* genome contains 13 isoforms (DELILLE *et al.* 2001), while 6 are found in cotton (ZHANG *et al.* 2010), 8 in rice (YAO *et al.* 2007), and 5 in barley (SCHOONHEIM *et al.* 2007). The soybean genome encodes 16 transcripts of 14-3-3 (LI *et al.* 2011) while in tobacco 17 potential isoforms are present (KONAGAYA *et al.* 2004). 14-3-3 proteins form homo- or heterodimers with a molecular weight *per monomer* of approximately 30 kDa each. They interact with specific binding motifs of phosphorylated target proteins (JOHNSON *et al.* 2010), although 14-3-3 protein interactions with atypical or non-phosphorylated motifs have also been reported (WATERMAN *et al.* 1998, DU *et al.* 1996). The interaction with 14-3-3 proteins always affect either the activity or the cellular localization of target proteins (PAUL *et al.* 2012). The total number of partners interacting with 14-3-3s in all eukaryotes was estimated higher than 500 (KLEPPE *et al.* 2011). In plants, several studies have shown that 14-3-3 proteins bind and regulate enzymes of primary metabolism, whereas no evidence for 14-3-3 interaction with enzymes of secondary metabolism has been

reported (DHAUBHADEL *et al.* 2010). The secondary metabolism of plants produce anthocyanins synthesized via the flavonoid pathway. Besides a multitude of biological roles, anthocyanins contribute also to the colour of the grapes and are important indicators of maturity and quality of the berries (SPARVOLI *et al.* 1994). To date two 14-3-3 isoforms, called *VVI_CS* and *VV2_CS*, have been characterized in grape (FARACE *et al.* 2015), but at least seven other putative isoforms have been identified in the Grape Genome Browser (JAILLON *et al.* 2007). *VVI_CS* and *VV2_CS* are expressed in the berries with two different profiles depending on the developmental stages of the berries (FARACE *et al.*; 2015). This result suggested the possible interaction between *VVI_CS* and *VV2_CS* and enzymes involved in berry maturity. From this point of view, enzymes from flavonoid pathway and producing anthocyanins could be good candidates. Although the anthocyanin biosynthetic pathway has been identified in grape (BOSS *et al.* 1996), no data about the involvement of 14-3-3s in the production or the post-transductional regulation of the anthocyanin biosynthetic pathway is known.

In this work, we investigated the *in vitro* interaction of *VVI_CS* and *VV2_CS* proteins (FARACE *et al.* 2015) with two important enzymes of the flavonoid biosynthesis pathway: the anthocyanidin synthase (ANS) and dihydroflavonol-4-reductase (DFR). We show here for the first time that 14-3-3 proteins bind to anthocyanidin synthase and inhibit its enzymatic activity *in vitro*. Also, we performed an *in silico* analysis of ANS protein sequence to identify putative binding sites for 14-3-3 interaction.

Material and Methods

Oligonucleotides were obtained from Eurogentec (Belgium). His-binding resin was from Novagen (Darmstadt, Germany), thrombin and glutathione Sepharose 4B from GE Healthcare (Uppsala, Sweden). 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). PKA, alkaline phosphatase-conjugated secondary antibodies and other chemicals were of analytical grade and were purchased from Sigma (St. Louis, Missouri, USA).

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Vitis cDNA cloning and sequence analysis: the two full-length 14-3-3 coding sequences (VV1_CS, GenBank accession number ACO40495; VV2_CS, GenBank accession number ACO40494) were isolated and expressed in *Escherichia coli* (FARACE *et al.* 2015). The full-length coding sequence for the ANS protein (GenBank accession number GQ281057) was amplified from a post-veraison cDNA library of *Vitis vinifera* L. ('Cabernet-Sauvignon') using the following primers: ANS-sph1-F (ATTAGCATGCAGAATGGTGGACTTCAGTGG) and ANS-pst1-R (CATGACGTCTACTTTATGTTGTTCC).

The amplified gene product was ligated into pGEM-T Easy (Promega), sequenced and subcloned into the pQE-30Xa bacterial expression vector (Qiagen). Using this cloning strategy, six His-tag sequences carried by the vector were added in frame to the 5' end of the construct. The resultant plasmid was used to transform *E. coli* M15 strain (pREP4) (Stratagene). DFR (GenBank accession number AY780886.1) was identified and prepared as already described (PETIT *et al.* 2007).

Recombinant protein expression and purification: VV1_CS and VV2_CS 14-3-3 protein expression was made according to FARACE *et al.* 2015. ANS and DFR proteins were expressed in M15 *E. coli* as His-tagged proteins using pQE-30Xa vector and purified by affinity chromatography with His-Bind Resin (Novagen) as already described (PETIT *et al.* 2007).

Analytical methods: protein concentration was determined by the method of Bradford (BRADFORD 1976) using bovine serum albumin as the standard. Proteins isolated from *Vitis* berries were quantified by Lowry method (LOWRY *et al.* 1951).

In vitro phosphorylation of VVANS and VVDFR: phosphorylation of ANS and DFR was performed in a reaction mixture containing 20 mM Tris-HCl pH 7.5, 100 mM NaCl, 12 mM MgCl₂, 10 U protein kinase A, 10 mM ATP. The kinase reaction was initiated by the addition of 1.5 mg of recombinant ANS or DFR and incubation was conducted at 30 °C for 30 min. In order to verify phosphate incorporation, phosphorylation was performed in the presence of 1.11 MBq of [γ-³²P]ATP (110 TBq/mmol) and proteins were subjected to SDS-PAGE and to autoradiography.

Overlay assay: the ³²P-labeled VV1_CS and VV2_CS 14-3-3 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 Na₂HPO₄ and 1.8 mM KH₂PO₄, pH 7.3), beads were incubated in 30 μL of buffer A (20 mM Tris-HCl, 12 mM MgCl₂, 100 mM NaCl, pH 7.5) with 1.11 MBq of [γ-³²P]ATP (110 TBq/mmol) and 10 units of the catalytic subunit of protein kinase A 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 overnight at 22 °C. The mixture was centrifuged and the supernatant was collected. ³²P incorpora-

tion 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 according to FULLONE *et al.* (1998), with minor modifications (FARACE *et al.* 2015). Each overlay experiment was performed at least three times and similar results were obtained.

Affinity chromatography assays: VVANS and VVDFR, previously phosphorylated, were immobilized onto His-bind beads and incubated with purified VV1_CS and VV2_CS 14-3-3 proteins. After three washes with 4 volumes of 0.5x wash buffer (4 M NaCl, 480 mM imidazole, 160 mM Tris-HCl, pH 7.9) and after removing final wash, 4 volumes of 0.5x elute buffer (4 M imidazole, 2 M NaCl, 80 mM Tris-HCl, pH 7.9) were added. Resin-bound proteins were eluted and analyzed by immunoblotting with anti-14-3-3 antibodies as described below.

SDS-PAGE and Immunoblotting: SDS-PAGE was performed as described by LAEMMLI (1970) in a Mini Protean apparatus (Bio-Rad). For immunoblotting, samples of affinity purified 14-3-3 were subjected to SDS-PAGE and blotted onto nitrocellulose membrane using a semidry LKB apparatus (2 h, 0.8 mA·cm²). Blots were probed with anti-14-3-3 antibodies (1/500) (FULLONE *et al.* 1998). Proteins were revealed by alkaline phosphatase-conjugated secondary antibodies (1/10000) using enhanced chemiluminescence detection (ECL kit, GE Healthcare).

ANS activity and LC-MS analysis: The ANS activity was measured as described previously (WELLMANN *et al.* 2006) with purified ANS (50 mg) ± 14-3-3s (100 mg) and catechin (100 mM) as substrate (Sigma-Aldrich) in the presence of 1 mM MgCl₂. Catechin and catechin dimer produced were separated, identified and quantified by HPLC/ESI-MS analyses carried out on a Surveyor HPLC system coupled to a LCQ Advantage spectrometer equipped with an ion trap mass analyzer (ThermoElectron, San Jose, United States).

Statistical analysis: The data are presented as mean ± standard deviation. To determine whether there is a statistical significant difference ($p < 0.05$) between the data obtained for the ANS activity, a One-way Anova test (with the Tukey's Multiple Comparison Test, comparing all pair of data) were carried out using GraphPad Prism 5.

Bioinformatics study: The bioinformatics analysis was performed by analyzing all putative 14-3-3 binding sites on the ANS: RSX[**pS/pT**]XP (mode I), RXXX[**pS/pT**]XP (mode II), [**pS/pT**]X_{1,2}-COOH (mode III). [**pS/pT**] refers to a phosphorylated serine *or* treonine residue, X_(1,2) indicates a generic residue (that may be present once or twice). For a manual search of these pattern we used the tool PattInProt (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_pattinprot.html), and for an automatic search we used three popular software: ELM (DINKEL *et al.* 2013), Scansite (JOHNSON *et al.* 2010) and 14-3-3-Pred (MADEIRA *et al.* 2015). The program NetSurfP (PETERSEN *et al.* 2009) was used to predict the accessibility of the serine/treonine residues in the ANS sequence, and the exposition of these residues was visually verified using

the Cn3D program (WANG *et al.* 2000a) visualizing a 3D structure of an *Arabidopsis thaliana* ANS (Genbank accession CP002687) similar to VVANS.

Results

VV1_CS and VV2_CS 14-3-3 proteins interact with VVANS: In order to investigate the potential role of 14-3-3 proteins during berry development and ripening, the ability to interact with VVANS and VVDFR was first tested by *in vitro* overlay and pull-down assays.

In the overlay assay, recombinant VVDFR and VVANS from grape were expressed in *E. coli* as His-tagged proteins and purified by affinity chromatography. Since phosphorylation of the target protein usually is a prerequisite for 14-3-3 binding, the two recombinant enzymes were phosphorylated *in vitro* by protein kinase A (PKA). The incorporation of phosphate was visualized through the use of [γ - 32 P]ATP as substrate. The autoradiography (Fig. 1) shows that PKA was able to phosphorylate both VVDFR and VVANS. Phosphorylated and unphosphorylated DFR and ANS were subjected to overlay assay using 32 P-labelled VV1_CS and VV2_CS 14-3-3s as probes. Both VV1_CS and VV2_CS 14-3-3 proteins specifically interacted with phosphorylated VVANS (Fig. 2A, lanes 2), whereas no interaction was detected in the absence of phosphorylation (Fig. 2A, lanes 3). On the other hand, VV1_CS and VV2_CS did not interact with DFR, either phosphorylated or not (Fig. 2A, lanes 4-5).

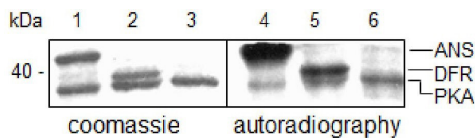


Fig. 1: *In vitro* phosphorylation of ANS and DFR. ANS (lane 1, 4) and DFR (lane 2, 5) were incubated with PKA in the presence of [γ - 32 P]ATP, separated by SDS-PAGE and subjected to Coomassie staining (left panel) and autoradiography (right panel). Phosphorylation was also carried out in the presence of PKA alone (lane 3, 6) as control.

To confirm the interaction between VVANS and 14-3-3 proteins, a pull down assay was carried out (Fig. 2B). Previously phosphorylated His-tagged VVANS was immobilized onto Ni $^{2+}$ -resin and incubated with the *E. coli* lysate expressing VV1_CS or VV2_CS 14-3-3 proteins. In the pull-down test, proteins bound to the matrix were eluted with imidazole buffer and subjected to immunoblotting with anti-14-3-3 antibodies. As shown in Fig. 2B, both VV1_CS and VV2_CS were found associated to ANS, appearing as bands of about 30 kDa. These two independent experiments clearly indicate that VV1_CS and VV2_CS proteins bind *in vitro* to phosphorylated ANS.

In silico analysis of consensus sequences in ANS: The *in silico* analysis was performed in order to identify phosphorylatable 14-3-3 binding sites present in ANS, according to the three consensus mo-

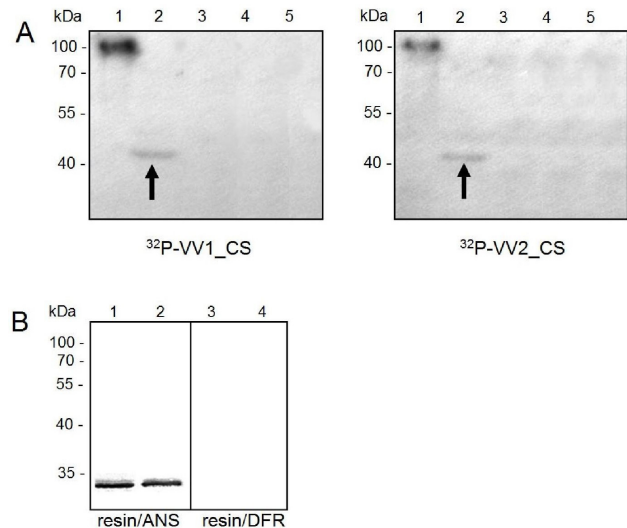


Fig. 2: Interaction of VV1_CS and VV2_CS with ANS and DFR. **A**): Overlay assay. ANS and DFR phosphorylated (lanes 2 and 4, respectively) or not (lanes 3 and 5, respectively) and maize root plasma membranes containing the H $^{+}$ -ATPase (lanes 1), as a positive control, were subjected to SDS-PAGE, transferred onto nitrocellulose membrane and incubated with 32 P-labelled VV1-CS (left panel) or 32 P-labelled VV2_CS (right panel). Radioactivity was detected by autoradiography. The arrows indicate that ANS separately bound to VV1_CS and VV2_CS. **B**): Pull-down assay. Previously phosphorylated His-tagged ANS and DFR were immobilized onto Ni $^{2+}$ -resin and incubated with VV1_CS (lanes 1 and 3) and VV2_CS (lanes 2 and 4). After washing, resin/ANS bound proteins were eluted by imidazole-containing buffer, separated by SDS-PAGE and subjected to immunoblotting with anti-14-3-3 antibodies. All these experiments were performed three times and a representative for each of them is shown.

tifs described for 14-3-3 protein interaction (JOHNSON *et al.* 2010). In Fig. 3, the peptide sequences of the ANS that may be sites of interaction with 14-3-3s are indicated. We searched for consensus sequences belonging to the mode 1 or the mode 2. Both modes contain a) a phosphorylatable amino acid, serine or threonine, and b) at least arginine or serine residue, respectively at position -3 and -2, and c) a proline at position +2, with respect to the phosphorylated residues. A third consensus sequence searched is SK-COOH (mode III), which corresponds to the pattern [pS/pT]X $_{1,2}$ -COOH.

By using PattInProt, 5 consensus sequences corresponding to the above presented features, *i.e.* RVEpSLS, SGIQpSIP, RSLApTKI TVSEpTEP and pSK-COOH were found (Fig. 3). These 5 consensus sequences have characteristics shared with other consensus sequences found in plant protein targets. In particular, the residues R (arginine), S (serine) and P (proline) are never simultaneously present in the mode but at least one of them is always present (JOHNSON *et al.* 2010, suppl. material 1). The sequence RVEpSLS has been indicated from EML as the most likely and TVSEpTEP has been considered the most likely by 14-3-3-Pred and Scansite. An analysis provided by NetSurfP, for the search of accessible residues to solvent on the ANS, shows that the most exposed serine/threonine residues are the T $_{326}$ (TVSEpTEP) and the S $_{354}$

MVTSVAPR**VeSLSSsGIQSI**PKEYIRPQEELTSIGNVFEEKKDEGPQVP 50
 TIDLKDI ESEDEVVRERCREELKKAAMEWGMHLVNHGISDDLINRVKVA 100
 GETFFNLPMEEEKYANDQASGKIAGYGSKLANNASGQLEWEDYFFHLIF 150
 PEDKRDMTIWPKTPSDYVPATCEYSVKL**RSLATK**iLSVLSLGLGLEEGRL 200
 EKEVGGMEELLQKKINYPKCPQPELALGVEAHTDVSALTFILHNMVPG 250
 LQLFYEGKWVTAKCVPNSIIMHIGDTIEILSNGKYKSILHRGLVNKEKVR 300
 ISWAVFCEPPKEKIIILKPLPE**tVSETEP**PLFPPrTFsqHIQhKLFRkTQE 350
 ALL**SK**-COOH 355

Fig. 3: *In silico* analysis of the ANS protein sequence to establish positions of 14-3-3 binding motifs. In grey block are indicated the five putative consensus sequences present in ANS. In bold are indicated the phosphorylable serine/threonine residues, in lowercase the mismatches compared to the "canonical" 14-3-3 motifs.

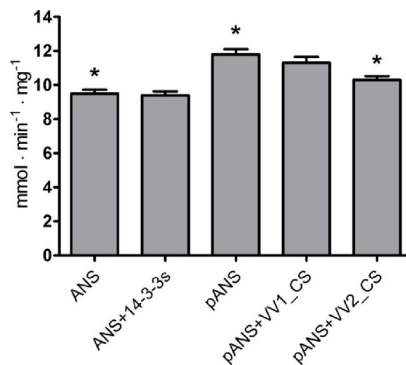


Fig. 4: Modulation of the ANS activity by 14-3-3s. The specific activity indicates the production of catechin by ANS. The activity is determined with the enzyme phosphorylated (pANS) or not. The activity was also tested with VV1_CS or VV2_CS in the reaction medium. All these experiments were performed three times. Asterisks indicate significance at $p < 0.05$ between pANS and ANS and between pANS and pANS+VV2_CS.

(pSK-COOH), but in general, all the proposed residues/patterns can be accessible to solvent/client (Table). A further visual check was made by showing the residual patterns in 3D models of an *Arabidopsis thaliana* ANS via the Cn3D program (WANG *et al.* 2000b). The result is that such residues are effectively exposed to solvent/client (data not shown).

In vitro modulation of ANS activity: In order to evaluate whether 14-3-3 interaction with ANS might modulate enzyme activity, an *in vitro* test was performed. As shown in Fig. 4, phosphorylation of ANS increases significantly its activity. The association of VV1_CS with the phosphorylated enzyme did not alter the enzyme activity, whereas the interaction of VV2_CS had a significant inhibitory effect. No effect was detected by incubating each 14-3-3 protein with the unphosphorylated ANS.

Discussion

14-3-3 proteins interact with a large number of protein targets (DE BOER *et al.* 2012), but so far an *in vivo* or *in vitro* interaction with enzymes of the secondary plant metabolism has not been reported. In grapevine, flavonoids are important secondary metabolites that are responsible for the colouring in the berry and its quality, which are crucial

Table

Protein surface accessibility of serine/threonine residues in ANS. This analysis was performed by NetSurfP. Column legend: aa_x: Amino acid in "x" position; CA: Class assignment ("B" for buried or "E" for Exposed - Threshold: 25 % exposure, but not based on RSA); RSA: Relative Surface Accessibility; ASA: Absolute Surface Accessibility

aa _x	CA	RSA	ASA
S ₁₁	E	0.343	40.188
S ₁₉	E	0.513	60.159
T ₁₈₃	E	0.322	44.675
T ₃₂₆	E	0.813	112.749
S ₃₅₄	E	0.701	82.110

factors that influence wine making. Two key enzymes of the biosynthetic pathway of flavonoids are DFR and ANS (Boss *et al.* 1996), that are directly involved in the anthocyanins production. To investigate the regulation of these two enzymes is important to understand the processes that are the basis of the colour and quality of the berry.

The first step was to find for a direct *in vitro* interaction between 14-3-3 and DFR and ANS. Phosphorylated and non-phosphorylated ANS and DFR were probed with the two recombinant *Vitis* 14-3-3 isoforms, via overlay and pull-down. Results showed that the 14-3-3s bound only to the phosphorylated ANS, no interaction with the DFR was detected. Afterwards, we tried to ascertain whether 14-3-3 interaction with ANS could result in modulation of enzyme activity *in vitro*. Interestingly, while the interaction with VV1_CS did not modify significantly the enzyme activity, the interaction with VV2_CS lowered its activity, revealing an inhibition of ANS by this 14-3-3 isoform. This inhibitory effect by 14-3-3 has already been reported for enzymes of the primary metabolism such as the nitrate reductase (SEHNKE *et al.* 1996).

ANS protein levels vary significantly during grape berries development and ripening, reflecting the relative abundance of corresponding mRNA: the ANS transcript has been demonstrated to strongly increase at *véraison*, accordingly with anthocyanins accumulation (WANG *et al.* 2011). It is interesting to note that, as previously demonstrated, the VV1_CS and VV2_CS mRNA levels decrease during the post-*véraison* stages of the berry (FARACE *et al.* 2015). Our preliminary test confirms that the abundance

of ANS and 14-3-3 proteins would reflect the mRNA levels observed in the post-veraison phase by WANG and colleagues and ourselves (data not shown). In this respect the *in vitro* observed 14-3-3 slight inhibitory effect on the ANS activity could represent a regulatory mechanism for fine tuning of anthocyanin synthesis. However, it must also be considered that *in vivo* the abundance of 14-3-3 proteins could be higher than that of ANS, thus resulting in a more marked inhibitory effect on the enzymatic activity compared to our *in vitro* tests.

Finally, we searched of sites of interaction with 14-3-3 with an *in silico* study. The 14-3-3 proteins recognize different consensus sequences in their target proteins, which can be phosphorylated or not. The 14-3-3s may recognize, in their target proteins, the two classical patterns RSX[**pS/pT**]XP (mode I) and RXXX[**pS/pT**]XP, (mode II) where [b>pS/pT] shows a phosphorylated serine or threonine, with preference for certain amino acids at positions X. The third mode, like the classical Y**pTV** (FUGLSANG *et al.* 1999) is indicated as mode III with the general nomenclature [b>pS/pT]X_{1,2}-COOH (GANGULY *et al.* 2005). However, it must be underlined that the presence of an arginine, followed by a serine not phosphorylated in mode I and the presence of a proline in modes 1 and 2, although it is strongly favoured, is not required for the 14-3-3 binding to the target protein (ZHANG *et al.* 1997). In other cases (MASTERS *et al.* 1999, VINCENZ *et al.* 1996) atypical patterns exist containing a phosphorylated serine, such as KGQST**pSRG** (WATERMAN *et al.* 1998), or not phosphorylated, such as GHSL (DU *et al.* 1996). This makes it difficult to identify only *in silico* putative target sequences of 14-3-3s.

In the *in silico* analysis of the ANS sequence (Fig. 3), in agreement with results presented in Fig. 2, where both *Vitis* 14-3-3s interact with the phosphorylated ANS, we have searched only serine and threonine phosphorylatable patterns and identified five putative binding sequences: RVE**pSLS** (mode I), SGIQ**pSIP**, RSLA**pTKI**, TVSE**pTEP** (mode II) and **pSK**-COOH (mode III). All these patterns have variations from the canonical consensus sequences, but this is true also for other plant 14-3-3 binding targets (JOHNSON *et al.* 2010).

Conclusions

VV1_CS and VV2_CS 14-3-3 proteins interact with ANS and modulate its activity *in vitro* suggesting a possible role for 14-3-3 proteins in the regulation of plant secondary metabolism pathways. Further studies will be necessary to confirm this association *in vivo* and to identify the effective site involved in the interaction with 14-3-3 proteins.

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