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ORIGINAL PAPER

VvCO and *VvCOL1*, two *CONSTANS* homologous genes, are regulated during flower induction and dormancy in grapevine buds

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Abstract Two previously uncharacterized *Vitis vinifera CONSTANS*-like genes (*VvCO*, *VvCOL1*), which are predicted to encode proteins with homology to members of the *Arabidopis* CONSTANS family, were identified. Under controlled conditions, both genes show a diurnal expression pattern with peak at dawn. During grapevine bud development, *VvCOL1* is mainly expressed in dormancy, suggesting a participation in the transcriptional photoperiod control of bud dormancy induction and maintenance in this species. On the other hand, *VvCO* expression in latent buds is in agreement with a function during flowering induction. A spatial and temporal relationship in the expression of *VvCO*, *VFY* and *VvMADS8* (the *Arabidopsis LEAFY* and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* orthologues) in latent buds is observed, suggesting that these genes are involved in the seasonal periodicity of flowering in grapevines. Furthermore, our results provide a new molecular insight into tendril development showing that grapevine *CO* homologues are also expressed in this distinctive organ.

Keywords CONSTANS · Dormancy · Flowering · Photoperiod · *Vitis vinifera*

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Introduction

Grapevine (*Vitis vinifera* L.) is a woody perennial crop with a peculiar mode of reproductive development, which differs significantly from *Arabidopsis* and annual species. In temperate regions, grapevine sexual reproduction occurs over two growing seasons separated by a dormancy period. During the first growing season, the shoot apical meristem generates an extra-axillary structure within latent buds called the "uncommitted primordia" (UP). An UP will generate an inflorescence or a tendril primordia and their fate is determined in part by environmental conditions in which the plant develops (Mullins et al. 1992). The first 1–3 UP formed around summer will often undergo repeated branching and develop into immature inflorescences (flowering induction) establishing the number of inflorescence primordia per bud (fruitfulness) before dormancy. The bud burst occurs in the following spring and then the immature inflorescences continue differentiation to form floral organs on floral initials to produce flowers (Sreekantan and Thomas 2006). Bud fruitfulness is an economically important trait, because it affects directly the potential crop yield as well as the plant architecture and type of pruning employed. Variations in bud fruitfulness would be in part responsible for the inconsistent yields observed among years (up to 38% of variation), converting the induction and initiation stage of inflorescence within the latent bud in a relevant process for industry interests (Carmona et al. 2008).

Pioneering physiological studies through the 1960s–1970s identified the major environmental conditions that determine the outcome of UP as inflorescence primordia. Temperature and light intensity are considered the most important (Srinivasan and Mullins 1981), although grapevine bud fruitfulness is also affected by photoperiod, being

greater under long days than in short days (Buttrose 1969, 1970). Furthermore, American species of *Vitis* are more sensitive to day length than *V. vinifera* L. For example, Delaware vines (*Vitis* × *labruscana*) grown in long days can form nearly three times more inflorescence than those grown in short days, irrespective of the temperature regime (Mullins et al. 1992). However, the molecular basis of these photoperiod responses is unknown.

Photoperiod flowering regulation in angiosperms has been investigated in a number of plants revealing the conservation of molecular mechanisms in both dicots and monocots (Yanovsky and Kay 2003). In A*rabidopsis,* the day length flowering responses are mediated by the so-called "photoperiod pathway". In this pathway, day length is perceived by photoreceptors and, along with entrainment factors, it synchronizes an endogenous circadian clock with the environment. Changes in day length are detected by this system and translated into flowering time information via cyclic expression of *CONSTANS* (*CO*) (Putterill et al. 1995). In the photoperiod pathway, CO protein plays a central role by mediating the circadian clock and the floral integrators by positively regulating *FLOWERING LOCUS T* (*FT*) and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*) which promote the expression of genes specifying flower meristem identity (Suarez-Lopez et al. 2001; Searle and Coupland 2004; Turck et al. 2008). Furthermore, Lee et al. (2008) recently showed that *SOC1* regulates directly the expression of *LEAFY*, linking the flowering induction with floral development.

In grapevine, studies on the molecular basis of flowering induction process are scarce, even though homologues of the *Arabidopsis* floral integrators *LEAFY* (*VFY*), *FT* (*VvFT*) and *SOC1* (*VvMADS8*) have been described (Calonje et al. 2004; Carmona et al. 2002, 2007; Sreekantan and Thomas 2006; for recent review, see Carmona et al. 2008). Nevertheless, although genes involved in classical flowering pathways such as photoperiod can be found in the grapevine genome, their roles remain largely unknown. Therefore, in order to understand the genetic and molecular mechanisms of flowering in grapevine, we isolated and characterized two *CONSTANS*-like genes, *VvCO* and *VvCOL1*, from *V. vinifera* cv. Carménère. As a first step to understand their developmental role, we determined their expression patterns during two growing seasons covering important developmental events such as flowering induction, dormancy and flower and fruit development. When analyzed under different photoperiod conditions, both genes displayed a diurnal expression pattern. In addition, we show that *VvCO* expression is spatially and temporally coordinated with *VvMADS8* and *VFY* (the *Arabidopsis SOC1* and *LEAFY* orthologues, respectively), suggesting a role for these genes in the seasonal periodicity of flowering and possible conservation of a regulatory network similar to the *Arabidopsis* photoperiod pathway in grapevine buds.

Materials and methods

Plant material

Grapevine (*Vitis vinifera* L. var. Carménère) organ samples from specific developmental stages were collected from a commercial vineyard in the Maule Valley, Chile, during the 2006–2007 seasons. Buds were sampled monthly during two growing seasons from November 2006 to September 2007. During the first growing season, young buds in leaf axils (latent buds) were collected from November to March. Dormant bud samples were taken from April to August. In the second growing season, swelling buds were collected (September). Further in development, when new canes begun to grow, other organs were also collected: leaves, shoots, tendrils, flowers and berries in three stages of development (green, véraison and post-véraison or harvest time berries). Sampling was performed at approximately 10:00 a.m. to reduce the possibility of differences in gene expression due to circadian oscillation, as the plants were grown under field conditions.

Photoperiod analyses were carried out on *V. vinifera* L. cv. Carménère plants grown in pots that were generated from dormant cuttings which had been cane-pruned from the vineyard. The hardwood cuttings were placed in a heated bed with a 25°C base temperature for rooting. After rooting, the cuttings were planted in 1-l pots and transferred to a growth chamber at 25°C.

Cloning of *VvCO* and *VvCOL1*

To isolate the full length cDNA and genomic *COL* sequence from grapevine, a set of primers was designed based on the nucleotide sequence from an EST present in the NCBI/GenBank Database CAO49246 (*VvCO*) and in a private database (*VvCOL1*). PCR was performed with the primers: *VvCO-F*: 5′-ATGTTGAAGGACGAAGGTTGTA ACG-3′, *VvCO-R*: 5′-TTAGAATGACGGGACAATGCC ATATCC-3′; *VvCOL1-F*: 5′-ATGGTTGTTGAAGTGG AAAGTTGG-3′, *VvCOL1-R*: 5′-TCAAAACGATGGAAC GACGCC-3′ using 100 ng of leaf cDNA or genomic DNA as templates and 1.5 mM $MgCl_2$, 1× PCR buffer, 250 nM of each primer, 200 nM dNTPs and 0.25 U *Taq* polymerase (Invitrogen, CA, USA) in the reaction mixture. PCR parameters were: initial denaturation at 95°C for 10 min, 35 cycles of 95°C for 40 s, 58°C for 60 s and 72°C for 60 s and a final extension at 72°C for 5 min. The resulting DNA fragments were separated in agarose gels, cloned in the

pGEM-T vector (Promega, WI, USA) and subsequently sequenced.

Gene expression analysis

For *VvCO* and *VvCOL1* expression analyses, three independent total RNA extractions (biological replicas) were made from grapevine buds, vegetative and reproductive organs according to Chang et al. (1993). Total RNA integrity was verified by formaldehyde agarose gel electrophoresis and their purity by OD_{260}/OD_{280} nm absorbance ratio >1.95. Following DNase treatment of total RNA, first-strand cDNA synthesis was carried out from 2 μg of total RNA for each sample using oligo(dT) according to the manufacturer's instructions (Thermoscript RT-PCR System, Invitrogen). Transcript levels of genes were measured by quantitative PCR (qPCR) using a DNA Engine Opticon 2 Cycler System (MJ Research, USA). All reactions were performed using the Brilliant SYBR Green Master Mix (Stratagene, USA) according to the procedure described by the manufacturer. For each biological replicate, qRT-PCR reactions were carried out in triplicate (technical replicates) using 2 μl Master Mix, 0.5 μl 250 nM each primer, 1 μl diluted cDNA and nuclease-free water to a final volume of 20 μl. Controls (with no cDNA and RNA without RT) were included in each run. Fluorescence was measured at the end of each amplification cycle. Amplification was followed by a melting curve analysis with continual fluorescence data acquisition during the 65–95°C melt. The raw data were manually analyzed and expression was normalized to *V. vinifera GADPH* gene (*VvGADPH*, NCBI/GenBank Database accession number CN938023) and *UBIQUITIN* gene (*VvUBQ*, TIGR Database accession number TC32075) to minimize variation in cDNA template levels. *VvGADPH* and *VvUBQ* were selected for normalization due to its consistent transcript level throughout buds, vegetative or reproductive tissues. For each gene, a standard curve was generated using a cDNA serial dilution, and the resultant PCR efficiency calculations were imported into relative expression data analysis. To ensure that the transcripts of single genes had been amplified, qRT-PCR amplicons were sequenced and confirmed as the expected plant DNA sequences. Error bars shown in qRT-PCR data represent the mean ± SD of three biological replicates qRT-PCR reactions.

The primers used for each gene analyzed were the following:

VvCO Fw: 5′-GATTGTTGTTCGGTGGGGAGGTG-3′, *VvCO* Rv: 5′-GGCGGCTGCTGCTGATTATACTG -3′; *VvCOL1* Fw: 5′-CATTGGTGAACGACAACTGC-3′, *VvCOL1* Rv: 5′-CGACTCCCACTTCTAAAGAGG-3′; *VvMADS8* Fw: 5′-ACCAACAACTACAAGACTACC GAAC-3′, *VvMADS8* Rv: 5′-ACCTTCTCCTAAGAGT TTCCGTTTG-3′; *VFY* Fw: 5′-GACACCGAAGGTGGGG ATGAAC-3′, *VFY* Rv: 5′-CTCTCCTGGCTCCGTCA CTATG-3′; *VvGAPDH* Fw: 5′-TTCCGTGTTCCTA CTGTTG-3′, *VvGAPDH* Rv: 5′-CCTCTGACTCCTCCTT GAT-3′; *VvUBQ* Fw: 5′-GTGGTATTATTGAGCCAT CCTT-3′, *VvUBQ* Rv: 5′-AACCTCCAATCCAGTCATC TAC-3′.

Phylogenetic analysis

To identify the related sequences in the GeneBank database (http://www.ncbi.nlm.nih.gov/BLAST), protein–protein BLAST was performed using the deduced amino acid sequence for grapevine *CO* homologue. Partial or incomplete sequences were not included. ClustalX was used to create multiple alignments. The resulting alignment was used to assemble the phylogenetic tree by the neighbor-joining method (Saitou and Nei 1987) using MEGA version 3.1 (Kumar et al. 2004). Search for intron number and position was performed using the PlantGDB's GeneSeqer software (http://www.plantgdb.org/cgi-bin/GeneSeqer/index.cgi) comparing the mRNA and genomic DNA nucleotide sequences under strict conditions.

Photoperiodic regulation of *VvCO*-like genes

To determine the circadian regulation of the grapevine *COL* transcripts, grapevine potted plants were grown in cabinets in either continuous light (CL), short days (SD, 8 h light, 16 h dark) or long days conditions (LD, 16 h light, 8 h dark). Light intensity of 800 μmol m^{-2} s^{-1} was provided by high pressure sodium lights (OSRAM SON-T 400 W lamps). The cabinet temperature was maintained at 25°C. For SD and LD treatments, leaf samples from at least 15 grapevine plants were collected every 4 h for a 44 h period at the second week of treatment and pooled. For CL experiments, plants entrained in LD were transferred to CL conditions and the leaf samples were collected as in LD and SD tests. Total RNA was isolated and used for qRT-PCR expression analysis as described previously.

In silico analysis of the *VvCO* and *VvCOL1* promoter regions

Search for regulatory elements in the *VvCO* and *VvCOL1* promoters was performed using two softwares: the PLACE signal scan search provided by the PLACE database (http://www.dna.affrc.go.jp/htdocs/PLACE/; Higo et al. 1999) and the computer program provided by the PlantCare database (http://www.bioinformatics.psb.ugent.be/webtools/plantcare/html/; Lescot et al. 2002).

Results

Two functional *CONSTANS*-like genes are present in the grapevine genome

Two previously uncharacterized grapevine *CONSTANS*-like cDNAs (*VvCO* and *VvCOL 1*) were isolated. *VvCO* and *VvCOL1* genes show an open reading frame of 1,176 bp and 1,044 bp and are predicted to encode a protein of the 391 and 347 amino acid residues, respectively (Fig. 1). Analysis of the protein domain architecture shows, in both proteins, the presence of two tandem Zn-finger B-box motifs with a striking cysteine residues arrangement at the amino terminus and a CCT domain (CONSTANS, CONSTANS-like, TIMING OF CAB EXPRESSION 1) at the carboxyl end. Each Zn-finger B-box motif contains four cysteines in a C-X2-C-X16-C-X2-C arrangement. On the other hand, the CCT motif of about 45 amino acids long is rich in basic amino acids. In addition, a glycine-rich region consisting of five residues in a row (poly-G) was found in VvCO before the zinc-finger regions whereas an alanine and glutamine-rich regions (poly-A and poly-Q, respectively) were found in VvCOL1 following the zinc-finger regions (Fig. 1). Moreover, both proteins contain five valine-proline (VP) domains similar to those found in *Arabidopsis* CONSTANS-like 3 protein (Datta et al. 2006) (Fig. 1). In addition, a six amino acid motif (consensus G-I/V–V-P–S/T-F), which is typically found in *Arabidopsis* CO and COL1-5 proteins (Griffiths et al. 2003), was observed at the carboxyl end of both proteins.

To ascertain the genome organization of *VvCOL1* gene the corresponding genomic sequence was amplified by PCR. The genomic PCR product showed that *VvCOL1*

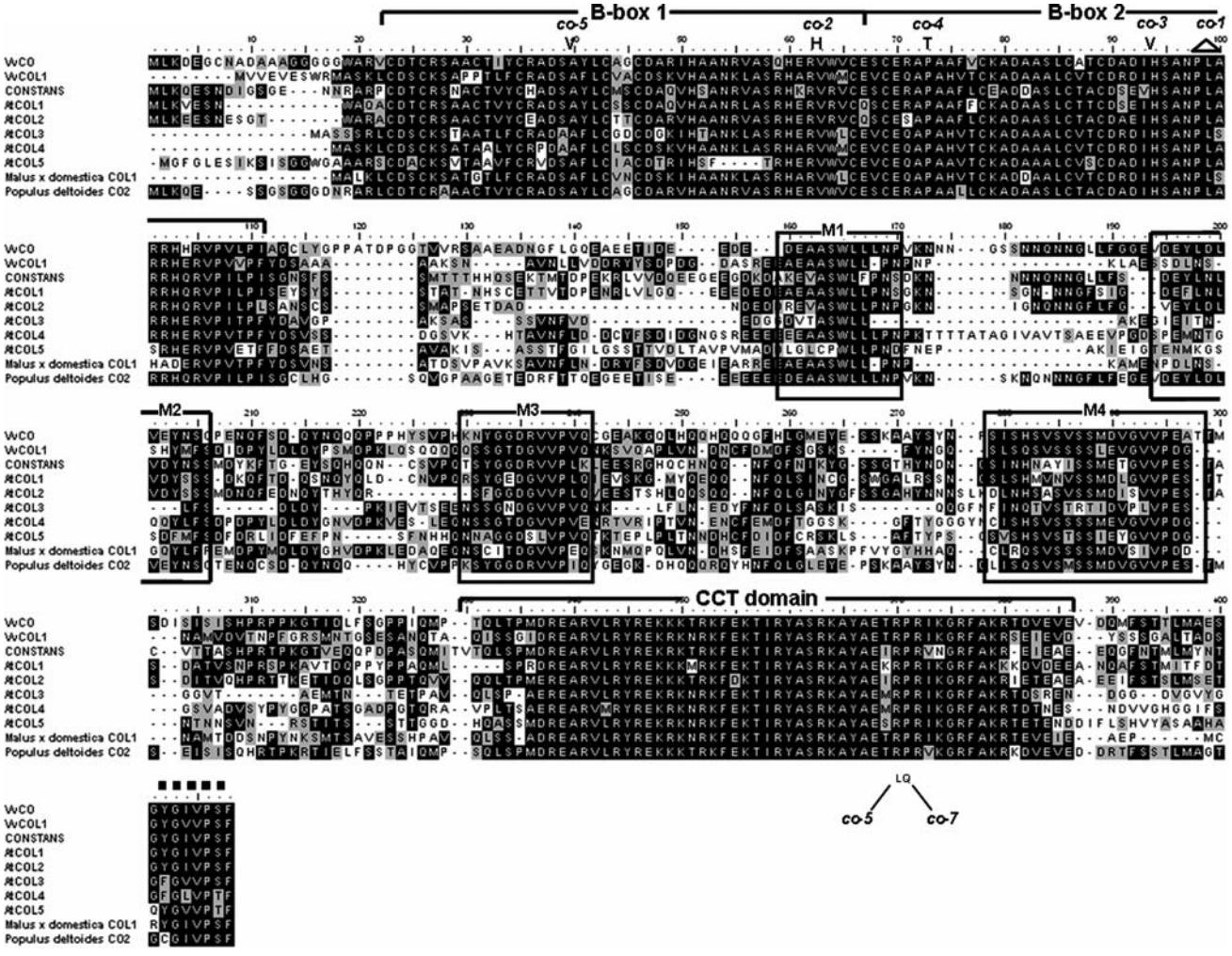

Fig. 1 Comparison of predicted amino acid sequence of VvCO, VvCOL1 and homologues from others plant species. Identical and similar amino acids among predicted protein sequences are shaded in *black* and *gray*, respectively. Residue changes in *Arabidopsis co* mutants are specified. The carboxyl end motif is upper-lined with a *dashed line*. B-boxes, CCT domain and middle conserved regions (M1–M4) are also indicated

contains two exons and one intron of 345 bp, placed between the B-boxes and the CCT domain similar to the *Arabidopsis CO* gene (data not shown).

VvCO and VvCOL1 are phylogenetically clustered with similar proteins from woody species

When the VvCO- and VvCOL1-predicted amino acid sequences were compared to sequences in the GeneBank database, they revealed significant similarities with other COL proteins of both woody and herbaceous dicots. VvCO was most similar to *Populus deltoides* COL proteins 1 (72% identity, gb|AAS00054.1|) and 2 (71% identity, gb|AAS00055.1|) whereas it showed a 54% of identity with CONSTANS from *Arabidopsis* (NP_197088.1). On the other hand, VvCOL1 was most closely related to *Malus domestica* COL proteins 1 (73%; AAC99309) and 2 (72%; AAC99310). It also showed high level of similarity with *Arabidopsis* COL 4 (66%) whereas only a 43% of identity with CONSTANS (NP_197088.1).

The multiple alignments of VvCO and VvCOL1 with the CO-like homologous proteins from *Arabidopsis* and other herbaceous and woody angiosperms revealed a high conservation at the B-box and CCT domain amino acid sequences in the grapevine homologue. Also, a high degree of similarity was found at the carboxyl end of the analyzed proteins (Fig. 1). Analyses of mutant *co* alleles in *Arabidopsis* have identified amino acid residues that are essential for CO functionality, both within the B-boxes and in the CCT domain (Robson et al. 2001). In VvCO and VvCOL1, these essential amino acid residues are conserved (Fig. 1). On the other hand, clearly the middle sections of the compared CO-like proteins were the most divergent, even though it is possible to identify four small similar regions (Fig. 1). The *Arabidopsis CO* and *COL* genes have been subdivided in three broad groups based on their structure and architecture of the proteins codified by them (Robson et al. 2001). Based on the overall architecture, VvCO and VvCOL1 are included in the Group 1, which comprises proteins that contains 2 B-boxes, one CCT domain and a carboxyl terminal motif as CO and AtCOL1-5. This group has been further subdivided based on the number and sequence characteristics of the conserved domains in their middle region (M1-4 domains) in: group 1a, comprising CO/AtCOL1/AtCOL2, and group 1c, comprising AtCOL3/AtCOL4/AtCOL5 (Griffiths et al. 2003; Zobell et al. 2005). Taken this classification into account, VvCO is more similar to the proteins from group 1a whereas VvCOL1 to group 1c.

To determine the evolutionary relationships of VvCO and VvCOL1 with other CONSTANS (CO) or CONSTANS-like (COL) proteins from angiosperms, a phylogenetic tree was assembled using the predicted protein sequences (Fig. 2). VvCO and VvCOL1 were clustered with the *Arabidopsis* CO and COL proteins that show 2 B-boxes, a CCT domain and a carboxy terminal motif (AtCO and AtCOL1-5) and separated from those that show only one B-box domain (AtCOL6-8) or a more divergent B-box Zinc finger (AtCOL9). Also is remarkable that VvCO was clustered with *Arabidopsis* CO, COL1 and COL2 whereas VvCOL1 was more related to COL3-5, possibly reflecting a functional divergence between the grapevine homologues. In addition, both grapevines CO-like proteins were highly related with homologues from woody angiosperms. Whereas VvCO protein was clustered with proteins from *Populus deltoides*, VvCOL1 protein was grouped with those from *M. domestica* (MdCOL1 and MdCOL2).

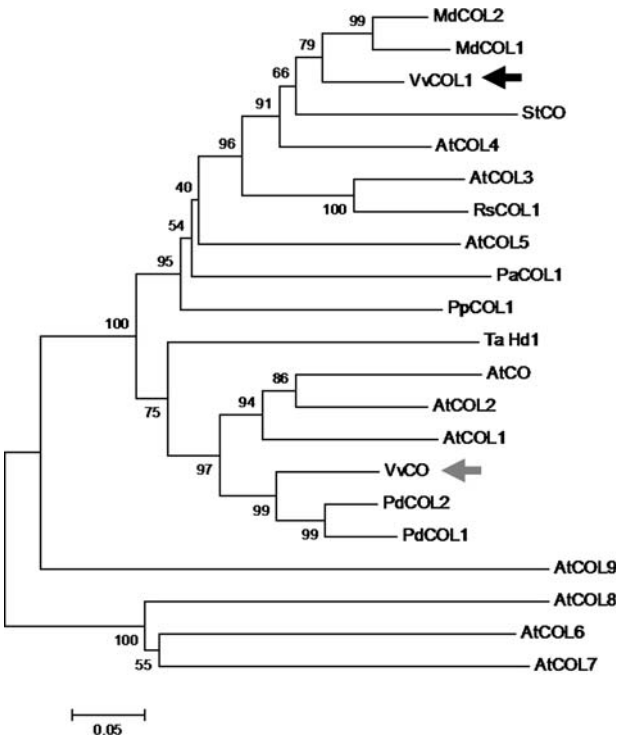

Fig. 2 Phylogenetic relationship among CO-like proteins. The proteins sequences presented in the multiple alignment of the Fig. 1 are included. The phylogram was generated by the MEGA 3.1 program from the multiple alignment of the deduced amino acid sequence from VvCO and VvCOL1 and homologous proteins from other plant species. Bootstrap values from 1,000 replicates were used to asses the robustness of the tree and are indicated. Accession numbers are the following: *Arabidopsis thaliana* CO (NP_197088.1); AtCOL1 (NP_197089); AtCOL2 (NP_186887); AtCOL 3 (NP_180052); AtCOL 4 (NP_197875|); AtCOL5 (AAM45054); AtCOL 6 (AAM10103|); AtCOL 7 (NP_177528|); AtCOL 8 (NP_175339); AtCOL 9 (NP_187422); *Malus domestica* COL 1 (AAC99309|) and 2 (AAC99310); *Populus deltoides* CO1 (AAS00054) and CO2 (AAS00055), *Solanum tuberosum* CO (ABH09237); *Rapahanus sativus* COL1 (AAC35496); *Physcomitrella patens* COL1 (BAD89084); *Triticum aestivum* Hd1 (BAC92734). The scale indicates the average substitutions per site

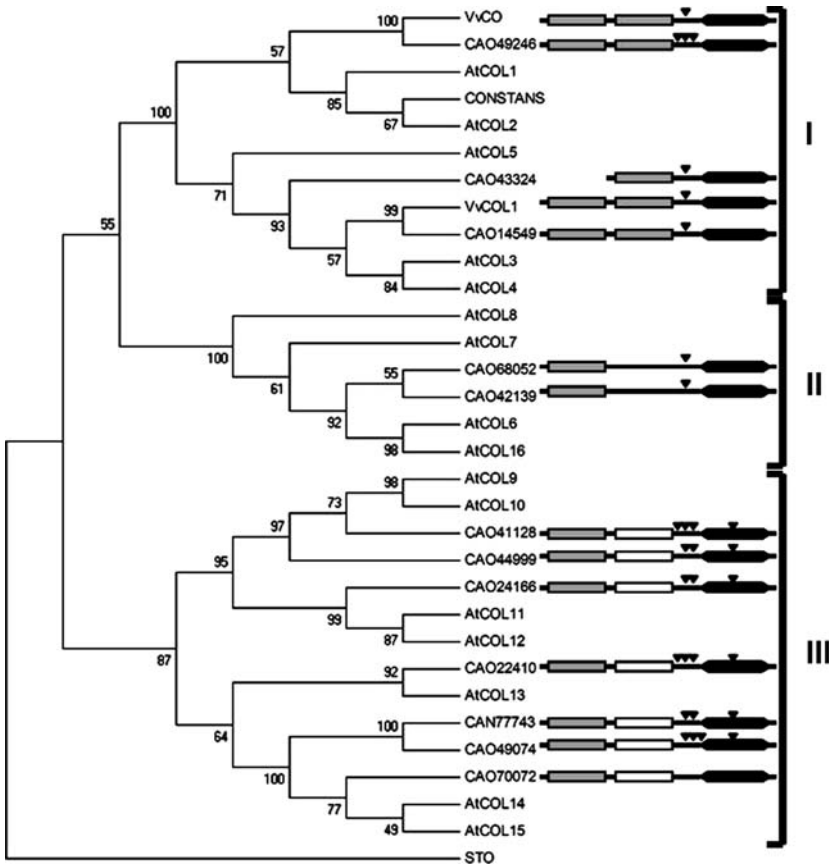

Fig. 3 Phylogenetic relationship among putative grapevine and *Arabidopsis* CO-like proteins. The three putative subfamilies (I, II, III) for the *Arabidopsis* and grapevine CO-like proteins are indicated. B-box domains are shown as *gray* and *white* boxes and CCT domains as polygonal black boxes. *Black arrowheads* specify the relative intron positions in each grapevine gene. The phylogram was generated by the MEGA 3.1 program. Bootstrap values from 1,000 replicates were used to assess the robustness of the tree and are indicated. Grapevine protein sequences were deduced from the nucleotide sequence obtained in this work (VvCO and VvCOL1) or from the GenBank accessions indicated. The *Arabidopsis* accession numbers are: CO and AtCOL1 to AtCOL9 (accessions numbers in Fig. 2); AtCOL10 (Q9LUA9); AtCOL11 (O23379); AtCOL12 (Q9LJ44); AtCOL13 (O82256); AtCOL14 (O22800); AtCOL15 (Q9C7E8); AtCOL16 (Q8RWD0) and STO (SALT TOLERANCE, NP_849598) as out-group

The three groups of the *Arabidopsis* CO family contain 17 members (CO and COL1-16) (Robson et al. 2001). The first group includes CO and COL1 to COL5 (two B-box proteins), the second group consists of COL6 to COL8 and COL16 (one B-box proteins), and the third group, COL9 to COL15 (one CO-like B-box and one more divergent zinc finger domain) (Griffiths et al. 2003). A first inspection of the grapevine genome sequence using VvCO and VvCOL1 or *Arabidopsis* CO-like peptides as queries, identified at least 12 putative proteins with homology to members of angiosperm CO family. To establish a relationship between the putative grapevine CO-like proteins and those from the *Arabidopsis*, a phylogenetic tree was developed. The tree shows that the grapevine CO-like proteins can be subdivided into three subfamilies as in *Arabidopsis* (Fig. 3). In *Arabidopsis*, proteins in groups I and II are encoded by genes that have a single intron located between the B-box and the CCT domain. Members of group III have a different gene structure, with three introns, two of which are between the B-box and the CCT domain and the third within the CCT domain. Structure of grapevine CO-like genes showed a general conservation of intron number and their relative position within each CO-like group. Despite that, some grapevine genes from Groups I and III present three introns between the B-box and the CCT domain (Fig. 3).

VvCO and *VvCOL1* are differentially expressed in vegetative and reproductive organs and show a daily expression pattern

As a first approach to ascertain the role of the *CONSTANS*-like gene in grapevine development, its temporal and spatial expression pattern was analyzed by qRT-PCR. Total RNA samples were isolated from buds, vegetative and reproductive organs in different but specific stages of

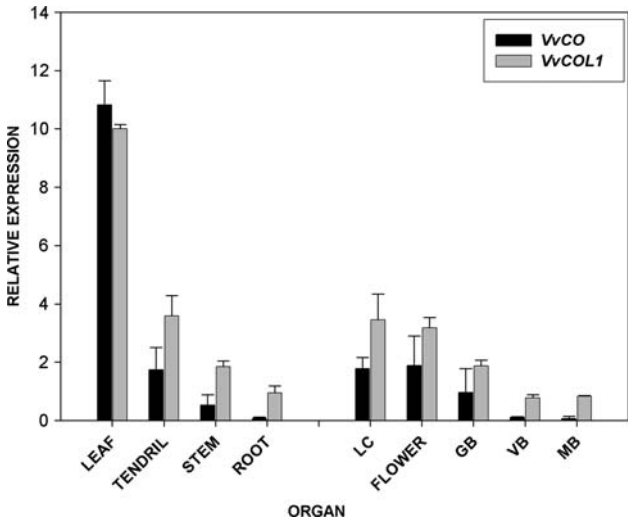

Fig. 4 Expression analysis of *VvCO* and *VvCOL1* in various organs of grapevine by qRT-PCR. The expression levels of *VvCO* and *VvCOL1* were normalized against that of *VvGAPDH*, using leaf as calibrator sample within vegetative organs (leaf, tendril, stem and root) and reproductive organs (*LC* little cluster, flower, *GB* green berry, *VB* veraison berry and *MB* mature berry at harvest time). Data are mean ± SD ($n = 3$)

development during two growing seasons covering important events such as flowering induction, bud dormancy and flower and berry development.

When the new cane begins to grow out, it differentiates both vegetative (leaves, shoot, tendrils and roots) and reproductive organs (flowers and berries). In these organs, *VvCO* and *VvCOL1* showed a similar expression profile, although *VvCOL1* expression level was in general relatively higher than that of *VvCO*. The analysis revealed that in vegetative organs, the *VvCO* and *VvCOL1* transcripts prevail mainly in leaves and in a lesser amount in tendrils, stems and roots (Fig. 4). In reproductive organs, *VvCO* and *VvCOL1* were mainly expressed in little cluster and flowers, whereas in berries, reduction of the transcripts of both genes were evident as the maturation stages progressed (Fig. 4).

In *Arabidopsis*, the *CO*, *COL1-4* and *COL9* genes show a daily pattern of expression which is circadian-controlled (Ledger et al. 2001; Cheng and Wang, 2005; Datta et al. 2006). In order to determine whether this attribute also affects *VvCO* and *VvCOL1* and because preliminary Northern hybridization experiments suggested that *VvCOL1* transcripts fluctuate during the day, *VvCO* and *VvCOL1* expressions were examined in dark/light cycles in more detail. Leaf samples were collected every 4 h over a 44 h period from grapevine plants grown in long day (LD) and short day (SD) conditions, and *VvCO* and *VvCOL1* transcript levels analyzed by qRT-PCR. Figure 5 shows that *VvCO* and *VvCOL1* transcript levels fluctuate daily, with the highest level at the beginning of the light period in both photoperiod conditions. Furthermore, under both photoperiod situations the lowest levels were found at the end of the illuminated period and a gradual increase was observed during the night. Because *VvCO* and *VvCOL1* expressions showed a daily oscillation pattern, the possibility that abundance of both transcripts was also affected by the circadian clock was further examined. Potted plants entrained in LD were transferred to continuous light (CL), leaves were harvested every 4 h until 44 h of initiation of the treatment. The results show a fluctuating behavior of *VvCO* and *VvCOL1* expressions under continuous light conditions. Interestingly, a period reduction of approximately 12 h for both genes was observed (Fig. 5). To gain information that could explain the expression behavior of *VvCO* and *VvCOL* under different photoperiod condition, an in silico search for cis-regulatory elements was performed with a 2,000 bp-far upstream sequence of each gene promoter. The analysis showed the presence of G-boxes (ACGT), Box I (TTTCAAA), Sp1 (CCG/ACCC), GATA (AAGGATAAGG) motifs and circadian-regulated elements (CAANNNNATC) in both promoters. Differences in the number and position of these motifs between both promoter sequences were evident. For example, the *VvCO* promoter presents four circadian-regulated elements at 1,698, 1,456, 856 and 801 bp from the start codon, whereas the *VvCOL1* promoter has only two of them at 1,667 and 806 bp from the start codon. The above-mentioned motifs are related to light responsiveness or have been involved in the circadian expression control in other angiosperm promoters (Castresana et al. 1987; Piechulla et al. 1998). Therefore, these cis-elements could be responsible for the light and circadian expression observed in *VvCO* and *VvCOL1*.

Grapevine *COL* genes are differentially expressed during bud development and *VvCO* expression is spatial and temporally coordinated with the grapevine *SOC1* and *LEAFY* orthologs in latent buds

During the bud development, *VvCO* and *VvCOL1* were expressed differentially (Fig. 6). In southernmost South American countries such as Chile, grapevine flowering induction occurs around the late spring and summer months within the latent bud placed on the shoot of the year. During this period, *VvCO* was highly expressed in axillary latent buds (Fig. 6) and further in latent bud development a gradual decrease in its transcripts level was observed. In dormant and second season woolly buds, these transcripts were relatively scarce. On the other hand, a slight increase of the *VvCOL1* transcripts was observed at the beginning and then also at the final state of the latent buds (Fig. 6). At the dormancy period, the expression of *VvCOL1* was relatively constant and no significant

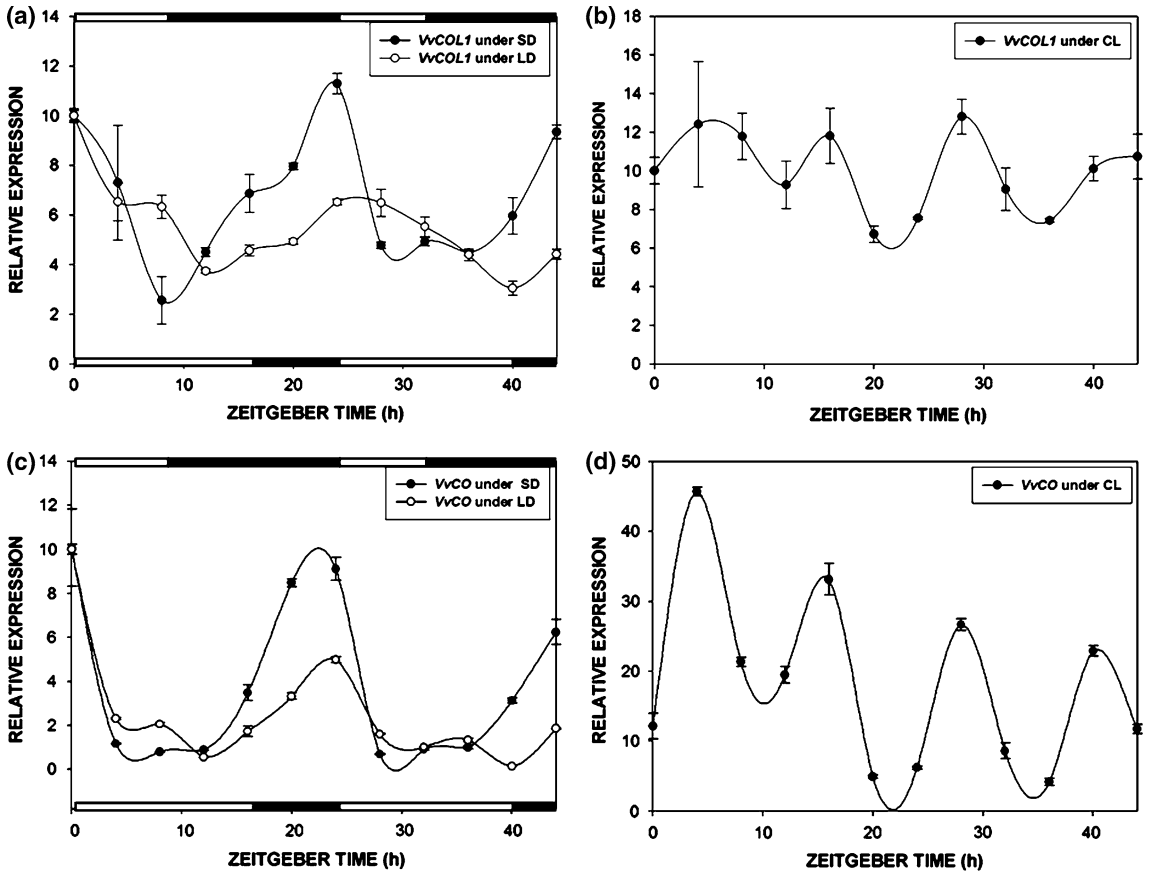

Fig. 5 Expression pattern of grapevine COL genes under different photoperiod situations. *VvCO* and *VvCOL1* expression profiles in long (*LD*) and short day (*SD*) photoperiods (**a**, **c**), and continuous light (*CL*) growing conditions (**b**, **d**). Leaves were collected at the different time points from at least 15 independent *V. vinifera* cv. Carménère plants. The expression levels of *VvCOL1* were normalized against that of *VvUBQ*. *Black boxes* indicate darkness and *white boxes* light

differences were observed. When the environmental conditions ameliorate due to spring arrival, grapevines resume their growth and bud burst occurs. At this stage, *VvCOL1* transcript levels diminished in the buds (Fig. 6).

In *Arabidopsis*, *CO* plays a central role in the photoperiod flowering pathway, mediating between the circadian clock and the floral integrators (Searle and Coupland 2004; Suarez-Lopez et al. 2001). Because *VvCO* was highly expressed in latent buds at the flowering induction period and to determine whether the expression behavior of *VvCO* fitted with the *Arabidopsis* flowering model, the expression of two putative grapevine floral integrator genes, *VFY* and *VvMADS8* (orthologues of the *Arabidopsis LFY* and *SOC1* genes, respectively), was also analyzed in buds (Carmona et al. 2002; Sreekantan and Thomas 2006). Maximum expression level of *VvCO, VvMADS8* and *VFY* was observed in latent buds at the flowering induction period. Moreover, a temporal relationship among these genes was evident. While *VvCO* was mainly expressed in November buds, peaks of *VvMADS8* and *VFY* were detected in December and January buds, respectively (Fig. 6), exhibiting a spatial and temporal relationship between the analyzed genes.

Discussion

The grapevine *VvCO* and *VvCOL1* are *CONSTANS*-like homologues

VvCO and *VvCOL1* encode proteins with two tandem repeated B-box zinc-finger domains at the amino terminus and a CCT domain at carboxyl end. These feature classifies them together with members of the subfamily I of *Arabidopsis* CONSTANS family (CO, COL1-5) (Robson et al. 2001; Griffiths et al. 2003), suggesting the conservation of this group of proteins in grapevines. Different number of *CONSTANS* family members appears to have evolved among plant species. While the *Arabidopsis CONSTANS* family is composed at least of 17 genes (Griffiths et al. 2003), preliminary grapevine genome analysis show the presence of at least 14 putative *CO*-like genes in the

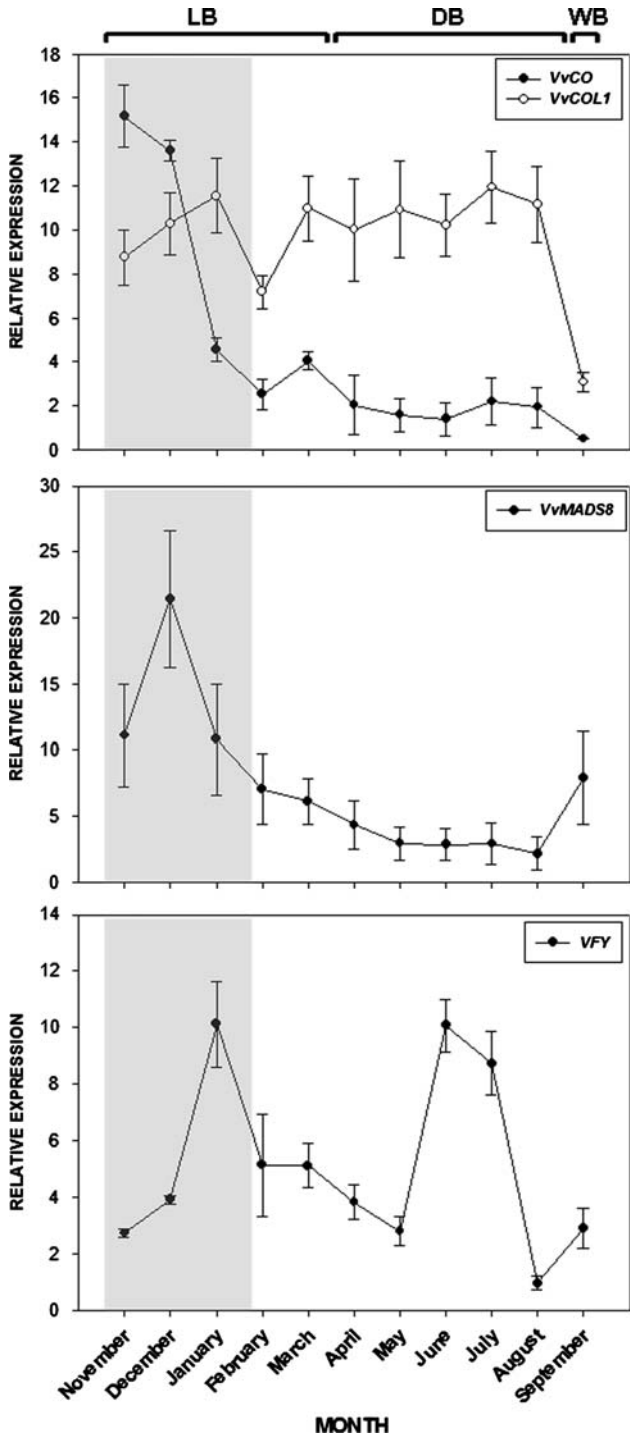

Fig. 6 Expression patterns of the *VvCO*, *VvCOL1*, *VvMADS8* and *VFY* genes in buds during two consecutive growing seasons. Latent buds (*LB*) in the first growing season, buds at dormancy (*DB*) and, second season or woolly buds at bud break (*WB*) were analyzed. The *gray area* indicates the period of the year when flowering induction occurs in Chile. The expression levels of analyzed genes were normalized against that of *VvGAPDH*. Each collection point was performed at the 14th–16th day of every month. Data are mean ± SD ($n = 3$)

grapevine genome. Furthermore, analysis of this multigene family shows that the major subgroups of CO proteins (I, II, III) described in *Arabidopsis* appear to have evolved in grapevine as well. Considering that grapevine belongs to the *Vitaceae* family, a basal family within the eudicots (Judd et al. 1999), the evolution of the CO family major subgroups in *V. vinifera* is in agreement with the hypothesis that this gene family preceded the monocot/dicot divergence (Griffiths et al. 2003).

Among other characteristics found in VvCO and VvCOL1, noteworthy are glycine, alanine and glutamine-rich regions. The prototype protein CO and its homologues from various plant species clearly differ in the number and length of these motifs. For example, the polyglutamine-rich motif found in VvCOL1 is shorter than the observed in the woody tree *P. deltoides* CO1 and CO proteins in the *Brassicaceae* family are apparently devoid of long glutamine-rich motif toward the C-end of the middle part (Drobyazina and Khavkin 2006). Plant amino acid rich motif length polymorphism may contribute to the genetic variation underlying adaptive evolution (Lindqvist et al. 2007). In addition, these repeats are potential transactivation sequences for protein–protein interactions that regulate transcription (Desveaux et al. 2000; Vaquero et al. 2000; Ding et al. 2006). In conclusion, the occurrence of the proteins in the functional CONSTANS I subfamily, emphasized by the conserved genomic organization, confirms the identity of the VvCO and VvCOL1 as a COL homologues.

VvCO and *VvCOL1* genes show a daily oscillation pattern of expression and both genes are expressed in tendrils

The diurnal oscillation exhibited by grapevine *COL* genes was strikingly different from the characteristic late evening (dusk) peak of *CO* and *Hd1* homologues. Instead, *VvCO* and *VvCOL1* transcripts peak at dawn, resembling the expression behavior of *AtCOL1, 2, 3* and *4* (Ledger et al. 2001; Datta et al. 2006). In addition, the amplitude of *VvCO* and *VvCOL1* transcripts was higher under SD than LD conditions differing from those reported for the *Arabidopsis* and ryegrass *CO* homologues, which are highly transcribed under long photoperiod conditions (Putterill et al. 1995; Martin et al. 2004). Besides, when grapevine plants were transferred to continuous light conditions, the *VvCO* and *VvCOL1* transcripts maintained a clear oscillation although a period reduction of approximately 12 h was observed for both genes. This is an interesting behavior because it differs from that of *Arabidopsis*, where rhythmic expression around 24 h of *CO, AtCOL1* and *AtCOL2* is

clearly observed under continuous light environment (Suarez-Lopez et al. 2001; Ledger et al. 2001). The loss of circadian rhythmicity has been reported for many eukaryotic systems (Lloyd and Murray 2007). This "dissociation" of rhythmicity sometimes uncovers ultradian rhythms (rhythms that cycle many times in a day and therefore measured in hours, minutes, seconds or even fractions of seconds). Ultradian rhythms have been associated to different functions in eukaryotes as signaling, optimization of responsiveness, provision of information for spatial and temporal organization (Lloyd 2006). Whether the transcriptional behavior of *VvCO* and *VvCOL1* in continuous light conditions is a particularity of these genes or is an essential property of grapevine clock mechanisms under "free running conditions" is a question that deserves attention. Further expression analysis of other grapevine *CO* homologues and circadian clock-related genes are necessary to address this issue.

When the new cane begins to generate different organs, *VvCO* and *VvCOL1* transcripts were independently analyzed. They were evidently predominant in leaves, but also, interestingly, detected in tendrils. Because previous studies showed that putative grapevine floral integrators and floral identity genes are also expressed in tendrils (Calonje et al. 2004; Sreekantan and Thomas 2006), this result agrees with the hypothesis that *Vitis* tendrils are modified reproductive organs adapted to climb, and suggests that a regulatory network similar to the photoperiod gene pathway could be operating for tendril development.

VvCO is principally associated with flowering induction whereas *VvCOL1* also with dormancy

With the onset of a cold season in temperate regions of Chile, the grapevine acquires a dormant state to survive the autumn and winter. Interestingly, during this period, the expression of *VvCOL1* was relatively constant (April–August) to finally reach a minimum at the bud burst in September (final of dormancy period). In *Vitis* species, dormancy may be induced by short day (SD) photoperiod, and in some varieties, a synergic effect with decreasing temperatures can be observed (Schnabel and Wample 1987; Fennell and Hoover 1991; Wake and Fennell 2000). Recently, Noriega et al. (2007) indicated that short days triggers the expression of a peroxidase RNA transcripts in *V. vinifera* cv. Thompson seedless buds. Taken this into account and the fact that *COL* genes have been involved in the photoperiod gating of dormancy transitions in woody angiosperms (Böhlenius et al. 2006), *VvCOL1* expression could suggest a role in mediating a transcriptional photoperiod control of bud dormancy induction and maintenance. However, because *Vitis* species demonstrate differences in responsiveness to SD photoperiod (Wake and Fennell 2000), further characterization of dormancy response to light cues and their relationship with *VvCOL1* or with other photoperiod related genes is necessary before general conclusions can be drawn.

VvCO expression was associated to seasonal flowering induction in latent buds (November and December) suggesting that the grapevine CONSTANS homologue could play a role during this process. In *Arabidopsis*, increases in the expression of *CO* are also associated with the induction of flowering. Similarly, in ryegrass and poplar (two perennial species) an increase of CO-like transcripts in leaves coincides with the flowering initiation period (Martin et al. 2004; Yuccer et al. 2002). Furthermore, a spatial and temporal relationship in the expression of *VvCO*, *VFY* and *MADS8* (the *Arabidopsis LFY* and *SOC1* orthologues) during the flowering induction in latent buds was observed, suggesting a role of these genes in the seasonal periodicity of flowering in grapevines. In addition, our data and the fact that the *FT* homologue is also expressed in latent bud stages in grapevine (Carmona et al. 2007), suggest the conservation of a regulatory network similar to the photoperiod gene pathway in *Arabidopsis* and a probable role of latent buds "per se" in the photoperiod perception and flowering induction in this species. Because grapevine buds are complex organs, in situ analysis of *VvCO* transcripts and complementation of *Arabidopsis co* mutant lines will provide a more accurately evidence of *VvCO* spatial expression and function.

It is known that environment × genotype interactions are responsible for bud fruitfulness and therefore for the large variation in yield observed in grapevine. Light is one of the most important environmental factors affecting various developmental stages of this crop (Carmona et al. 2008) and understanding the mechanisms involved in light perception can help us to develop genetic tools to control yield variation. This work constitutes the first attempt to dissect the grapevine CONSTANS family and the basis for further analysis of related genes involved in light and photoperiod perception pathways in this species.

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