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Molecular characterization and expression analysis of the Rab GTPase family in *Vitis vinifera* reveal the specific expression of a VvRabA protein

Philippe Abbal¹, Martine Pradal¹, Lisa Muniz¹, François-Xavier Sauvage¹, Philippe Chatelet², Takashi Ueda³ and Catherine Tesnière¹,*

¹ UMR1083, Sciences pour l'Œnologie, INRA, 2 Place Viala, F-34060 Montpellier cedex 1, France
² UMR1098, Développement et Amélioration des Plantes, INRA, F-34060 Montpellier, France
³ Department of Biological Sciences, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, J-113-0033 Tokyo, Japan

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Abstract

As a first step to investigate whether Rab GTPases are involved in grape berry development, the *Vitis vinifera* EST and gene databases were searched for members of the VvRab family. The grapevine genome was found to contain 26 VvRabs that could be distributed into all of the eight groups described in the literature for model plants. Genetic mapping was successfully performed; VvRabs were mostly located on independent chromosomes, apart from eight that were located on the as yet unassigned portions of the genome clustered in the ChrUn_Random chromosome. Conserved and divergent regions between VvRab protein sequences were identified. Transcript expression of 11 VvRabs was analysed by real-time quantitative RT-PCR. Except for VvRabA5b, transcript expression was detected, in general, in all the organs investigated, but with different patterns. In grape berries, VvRab transcripts were expressed at all stages of fruit development, with different profiles, except in the case of members of the A family which displayed generally similar patterns. The response to growth regulators in cell cultures was generally specific to each VvRab, with a differential pattern of expression for ethylene, auxin, and abscisic acid according to the VvRab. Interestingly, and unexpectedly considering transcript expression, western blotting using a monoclonal antibody raised against AtRabA5c (ARA4) showed a specific expression in the exocarp of ripe grape berries, in all seven red and white berry varieties tested. By contrast, no expression was detected in any of the other organs or tissues investigated. This paper contains the first description of Rab GTPases in *V. vinifera*. The involvement of a specific VvRab in grape berry late development and the potential role of this Rab GTPase are discussed in relation to literature data.

Key words: EST database, fruit ripening, genome mapping, grapevine phylogeny, quantitative RT-PCR, Rab GTPase, transcript level, unigene clustering, *Vitis vinifera*.

Introduction

Rab proteins constitute the largest branch of the Ras GTPase superfamily (Yang, 2002). They have been shown to be important regulators of the endomembrane traffic (Molendijk *et al.*, 2004), mediating communication between vacuole, plasma membrane, endoplasmic reticulum, Golgi, and cell wall. Members of this family are localized on distinct membrane compartments and exert functions in different trafficking steps. Rab GTPases regulate multiple aspects of vesicle trafficking, budding, and fusion through the binding of specific effector proteins (Wennerberg *et al.*, 2005; Grosshans *et al.*, 2006). The activity of these GTPases depends on their association with GTP or GDP. The active GTP-bound form activates downstream effectors and signalling pathways. The equilibrium between the active and the inactive forms of the small GTPases is controlled by positive and negative regulatory proteins in response to different cellular conditions and extracellular stimuli. Stimulations or interactions with partners activate the small GTPases, which will, in turn, stimulate several
downstream effector pathways, responsible for their biological effects.

In Arabidopsis, 57 Rab GTPase loci have been identified (Pereira-Leal and Seabra, 2001; Rutherford and Moore, 2002). This family is divided in eight subfamilies (RabA to H), which are themselves divided in 18 subclasses. Some subclasses are plant specific (Rutherford and Moore, 2002). Studies have shown the importance of some Rab GTPases in key steps of plant development, in particular, in processes such as pollen tube growth (De Graaf et al., 2005), or in the development and the ripening of fruits such as mango and tomato (Loraine et al., 1996; Zainal et al., 1996; Lu et al., 2001). In addition, it has been shown that expression of some Rab GTPases is modulated in response to concentration variations in phytohormones such as ethylene (Loraine et al., 1996; Moshkov et al., 2003a, b) or abscisic acid (Nishimura et al., 2004).

Fruit development involves a number of changes including cell division and enlargement, primary and secondary metabolisms with changes in colour, flavour, and texture (Brady, 1987; Seymour et al., 1993), together with the expression of a characteristic set of genes (Goes da Silva et al., 2005). Changes in colour, flavour, and texture are mediated by related enzymes which presumably are trafficked through the endomembrane system of the cell (Leah et al., 1995; Ono et al., 2006; Saint-Jore-Dupas et al., 2006), and transported to the vacuoles for pigments and volatile compounds and secreted to the apoplasm for cell-wall softening enzymes (Lu et al., 2001). In addition, variations of these changes are under the control of hormonal and stress signalling. It is a reasonable hypothesis therefore that the molecular switches that transduce signalling and control intracellular trafficking might be essential for these processes. Fruit is thus a pertinent and original model to investigate specific Rab GTPases functioning.

In grapevine (Vitis vinifera L.), members of the Rab family have been characterized for the first time in a perennial species. The Vitis genome being entirely sequenced (Jaillon et al., 2007), allowed us to retrieve VvRabs as exhaustively as possible at the present time. Transcript expression analysis of some of these VvRabs is presented in a developmental series of grape berries, in different grapevine organs, and in suspension cells in cultures. Representative samples were taken from various organs: leaves, tendrils, inflorescences, stamens, pistils, flowers, and pips. All samples were pooled from five different plants or cuttings (in the case of root samples) in order to randomize biological variations. A 2002 and 2006 series of 3 (early green stage) to 12 (fully ripe) weeks-post-flowering (WPF) berries were sampled (totalling eight stages) with 100 berries taken at each stage (20 berries from each plant from at least five bunches). Moreover, berries from different white berry varieties (Chardonnay, Riesling, Sauvignon), and red berry varieties (Alicante, Grenache, Merlot, Syrah, and Cabernet Sauvignon) were harvested at the ripening stage and exocarp was separated from the mesocarp. Samples were frozen in liquid nitrogen and stored at −80 °C.

Callus was initiated from stem fragments of in vitro-grown Cabernet Sauvignon plantlets on a solid induction medium composed of half-strength MS (Murashige and Skoog, 1962) macroelements, MS microelements, Morel’s vitamins, 1 g l⁻¹ casein hydrolysate, 20 g l⁻¹ sucrose, 5 μM NOA, 1 μM BAP, pH 5.8, and 7 g l⁻¹ agar. Cell suspension cultures were established from actively growing callus on a liquid medium composed of B5 macroelements (Gamborg et al., 1968), MS microelements, Morel’s vitamins (Morel et al., 1970), 250 mg l⁻¹ glutamine, 20 g l⁻¹

Materials and methods

In silico sequence analysis

To search databases for expressed sequence tag (EST) encoding VvRab sequences, five characteristic motifs were used to identify these sequences: (i) MGAYRAEYDYLFEVVLIDSGYKSNLLSRFTKNEFLESKSTIGVAFATSRIVDEK; (ii) KIDYVFKVVVGDSAAYGKTQITGQVQVFQVKIWDQGQYRAITSSAY-VRGAGVALLYVDHVTFTENVDRLKELHRDHTSSIV; (iii) DTAGQESFRSITRSYYGAAGALLYVDTIRRETFSNLASLWE-DAROHANPNMTMLG Nik; (iv) LGGLNAENGAAPDNKLRRV- KVLVLLDSGVGKSCIVLRFVRQDFPSKTGVGA; and (v) QTIALQDSTTVKFEIWDTAQGQYRAALPPLYRGAAVAVVVYDVSPE. Working with this set on NCBI resources, the sequences recovered were compared by TBLASTN to Genbank data. In order to get a unigene set, the EST sequences matching these five motifs were clustered and assembled using the Phrap software (http://www.phrap.org/phredphrap/phrap.html). Each member of this set was studied individually in order to check whether it represented or not a complete sequence, and whether its domain could qualify as an authentic Rab ORF domain, finally controlling the transcript for redundancy.

For phylogenetic analysis, sequence alignments, and comparisons of the 26 VvRabs nucleotide sequences with the ArRab proteins from A. thaliana were performed with ClustalW software (Thompson et al., 1994). An unrooted phylogenetic tree was generated using the Phylip Protein Sequence Parcimony Method (Felsenstein) software (http://bioweb.pasteur.fr/seqanal/interfaces/protpars.html), and was drawn using QuickTree and Treeview softwares.

To investigate the VvRab gene molecular organization, the VvRab full-length cDNA sequences were blasted (Altschul et al., 1997) against the Genoscope blast server for V. vinifera (http://www.cns.fr/cgi-bin/blast_server/projet_ML/blast.pl), resulting in several trace names corresponding to the best BLAST hits on the query sequence. Sequences related to these trace names and corresponding to the shotgun grapevine genome sequencing were obtained from the NCBI trace archive server (http://www.ncbi.nlm.nih.gov/Traces/trace.cgi). To evaluate the intron–exon organization, genomic sequences were aligned with the cDNA sequences and translated using the ExPaSy Proteomics server (http://www.expasy.org).

The genetic distribution of the V. vinifera Rab GTPase family was performed with cns software (http://www.genoscope.cns.fr/blat-server/cgi-bin/vitis/webBlat).

Plant material and treatments

The V. vinifera L. cultivar Cabernet Sauvignon was used in the form of field-grown vines, greenhouse-grown cuttings, and cell cultures. Representative samples were taken from various organs: roots, dormant buds, young expanding leaves, fully developed leaves, tendrils, inflorescences, stamens, pistils, flowers, and pips. All samples were pooled from five different plants or cuttings (in the case of root samples) in order to randomize biological variations. A 2002 and 2006 series of 3 (early green stage) to 12 (fully ripe) weeks-post-flowering (WPF) berries were sampled (totalling eight stages) with 100 berries taken at each stage (20 berries from each plant from at least five bunches). Moreover, berries from different white berry varieties (Chardonnay, Riesling, and Sauvignon), and red berry varieties (Alicante, Grenache, Merlot, Syrah, and Cabernet Sauvignon) were harvested at the ripening stage and exocarp was separated from the mesocarp. Samples were frozen in liquid nitrogen and stored at −80 °C.

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sucrose, 1 μM kinetin, 0.5 μM NAA, pH 6, according to Hawker et al. (1973). Suspension cells from 5-d-old subcultures (exponential growth phase; Torregrossa et al., 2002) were treated with 2-chloroethylyphosphonic acid (CEPA, 50 μM), an ethylene-releasing chemical (Abeles et al., 1992), 1-methylocyclopentene (MCP, 1 ppm), a specific inhibitor of ethylene receptors (Blankenship and Dole, 2003), abscisic acid (ABA, 50 μM) or α-naphthalene acetic acid (NAA, 50 μM). As ABA and NAA were dissolved in 1% ethanol, a corresponding control was also included. Before and 24 h after treatment, cell aliquots were collected on a Whatman filter by vacuum filtration, immediately frozen in liquid nitrogen, and stored at −80 °C. Two separate experiments were performed.

**RNA extraction and real-time quantitative RT-PCR analysis**

Total RNAs were isolated using the SV Total RNA Isolation System (Promega, France) for powdered suspension cells and using the Rneasy Plant Mini Kit (Qiagen) for all other tissues of 2006, while total RNAs from berries of 2002 were extracted on cesium chloride cushion (Tesniere and Vayda, 1991). First-strand cDNA was synthesized using 0.5 μg of total RNAs by priming with an oligo-dT-anchor at 42 °C for 50 min using a Superscript-II Reverse Transcriptase (Invitrogen) according to the manufacturer’s instructions.

Real-time quantitative RT-PCR was conducted with SYBR Green PCR Master Mix and gene-specific primers (Table 1). Each PCR reaction (20 μl final volume) contained 5 μl of template cDNA, 250 nM of each primer and 1× SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK). Thermocycling conditions were as follows: an initial enzyme activation of 10 min at 95 °C, followed by 40 cycles of denaturation for 15 s at 95 °C, annealing and extension for 1 min at 60 °C, with a final melt gradient starting from 60 °C and heating to 95 °C at a rate of 0.03 °C s⁻¹. The real-time PCR reactions were carried out in a 7300 Fast Real Time PCR System (Applied Biosystems, Warrington, UK). Fluorescence was measured at the 497 nm (excitation) and 521 nm (detection) wavelengths at the end of each extension step and at each 1 °C increment of the melt profile. Primer specificity was confirmed by analysing dissociation curves of the PCR amplification products.

All cDNA samples to be compared for transcript levels were analysed in triplicate for each gene in a single batch for each primer pair. To ascribe a relative transcript copy number to each cDNA sample, a purified PCR fragment of each gene sequence was serially diluted 10-fold to obtain template standards. The most concentrated standard was assigned an arbitrary transcript copy number and subsequent n-fold dilutions were accordingly assigned as relative copy numbers. Standards from 10⁻³ to 10⁻⁶ of the gene to be analysed and from EF1-α were included in the real-time PCR assay of cDNA samples. In each case, a dilution series of standards showed a linear change in cycle threshold values and cDNA templates were thus ascribed a relative transcript copy number by comparing their cycle threshold values with the standards. All templates and standards were run in triplicate and expressed as the average ± standard deviation. Sample values were corrected using the corresponding expression level of EF1-α, an isogene constitutively expressed (Terrier et al., 2001). The specificity of the PCR product generated for each set of primers was tested by cloning in pGemT-Easy (Promega, Madison, WI, USA) and sequencing (MWG, France) the product.

**Western blot analysis**

Powdered grapevine organs and berry tissues were suspended in 50 mM TRIS–HCl pH 7.5 extraction buffer containing 7 M urea, 2 M thiourea, 2% Triton X-100 (w/v), 2% PVPP (w/v), and 1% DTT (w/v) (Perugini and Schubert, 2002). Cell debris was removed by centrifugation, supernatant proteins were precipitated by TCA; pellets were then washed and proteins were solubilized as previously described (Abbal et al., 2007). After dilution (1/10), aliquots of the solution were used for protein content determination (Bradford, 1976). Equal amounts of protein (145 μg per lane for berries, 27 μg for organs) were separated by SDS-PAGE (14% acrylamide) along with molecular weight markers. Protein bands were transferred to nitrocellulose membranes by electrophoretic blotting as described in Abbal et al. (2007). Membranes were then incubated for 120 min at 4 °C with appropriate concentrations of antibodies (1:200). After rinsing three times for 10 min in PBS, membranes were incubated for 60 min at room temperature with secondary antibodies (1:2000) labelled with alkaline phosphatase (Sigma) in 50 mM TRIS–HCl pH 7.5 containing 150 mM NaCl. After rinsing with this last buffer, the blots were stained with 5-bromo-4-chloro-3-indolylphosphate toluidine salt–nitro-blue tetrazolium chloride (Sigma) following the manufacturer’s instructions. After staining, the reaction was stopped by the addition of PBS buffer with EDTA (2 mM).

**Mapping VvRab genes on the grapevine genome**

To locate VvRab proteins on the genetic map, the new grape genome data at http://www.genoscope.cns.fr/ was used. Each VvRab protein sequence was analysed using BLAT software. BLAT on proteins was designed to find sequences quickly with 80% (and greater) similarity to 20-(and over)-amino acid-long sequences. On DNA data, BLAT is designed to find sequences with 95% and greater similarity to 40 (and over)-base-long sequences. It may miss more divergent or shorter sequence alignments. It will

| Table 1. Primers 3’-end used for real-time quantitative RT-PCR experiments |
|-----------------|---------|-----------------|---------|-----------------|
| Pair name       | Primer 3’-end forward sequence | /stop codon | Primer 3’-end reverse sequence | /stop codon | Product length  |
| VvRabB1c F/R    | TGGATTTGTTGGATGGGATG             | +12        | GCCTATCTCCTAATGAGGCAAC         | +120       | 109 bp          |
| VvRabA1e F/R    | ACTTTCCCATTTTCCATGCA             | +42        | ATCTCTAGCTTTGGGGGGTTT          | +215       | 174 bp          |
| VvRabA2a F/R    | CCAGATTTGGGATTTAGT               | +50        | GTCAATTAGAAAAACCCCTCA          | +207       | 158 bp          |
| VvRabB5b F/R    | ATCAGAAGCAGCAGACAGCA             | +15        | ATGAACTCAGTGGAAACCTCT          | +164       | 150 bp          |
| VvRabA5c F/R    | TTTCTGTGTTGATCCTTCTC             | +22        | TGGCTGTGCCCTTCAAT             | +176       | 155 bp          |
| VvRabB1c F/R    | TACATTTGATTGGTTTCTTGGC           | +32        | ACTTTTCACTCAGGAGTGGC          | +131       | 100 bp          |
| VvRabB1d F/R    | TTTGAGATTCCCCATTTGGAATTG         | +3         | CGAATTCAAGAATACAGAAGG         | +164       | 162 bp          |
| VvRabC1 F/R     | TGATTATCTCTTAAATTCTTCTCC         | +4         | CATGACATGTGGTGGTTGGAA         | +93        | 90 bp           |
| VvRabD2c F/R    | TATTCTGTTGAGATGGTAG              | +8         | GAAACAGAAAAACATGAGGAGA        | +112       | 105 bp          |
| VvRabE1c F/R    | ATAATGGATGCAAAGGTCGT             | +13        | CCTAATGAGATGGAAAAACTACCCG     | +162       | 150 bp          |
| VvRabG3a F/R    | AATATGGATAGTGGAGACCTG            | +8         | AAAGTGCGCAACAGAACCA          | +153       | 150 bp          |
find perfect sequence matches of 33 bases, and sometimes find them down to 22 bases. The results clearly showed the best score for each matching chromosome, including its location. From these data, and using the CMAP software on the same server, it was possible to locate the closest markers on each side of the concerned area of the chromosome.

Results

VvRab cDNA sequence comparison

Twenty-six complete VvRab sequences were identified in the V. vinifera genome, and the characteristics of the unigene set are presented in Table 2. To address the existence of Rab-specific sequences among the VvRabs, the 26 known encoded proteins from V. vinifera were first aligned using the ClustalW algorithm (Thompson et al., 1994). VvRab sequences contained conserved regions existing in all members of the Ras superfamily (Fig. 1). Concerning the Rab-specific regions F1–F5, defined according to Pereira-Leal and Seabra (2000), F1 localizes to the effector domain, and F2 to a GTPase domain. Other regions (G and PM) are respectively involved in guanine and phosphate/Mg$^{2+}$ binding. Moreover, the presence of the double-cysteine motif in the C terminus like most of the VvRabs, is a very good diagnostic of a Rab protein. However, VvRabF1 lacked the C-terminal region containing part of the hypervariable region and the cysteine motif. It also had an extra stretch of amino acids at the N-terminus, which contains putative N-myristoylation and palmitoylation sites. All the VvRabs displayed subfamily-specific regions (SF) that mediate specific interactions between Rabs and different effectors. As presented in Table 3, the VvRab cDNAs encoded proteins of 200–239 residues, with predicted molecular masses around 24 kDa.

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Phylogenetic analysis of Rab GTPases expressed in V. vinifera

A comparison of the 26 deduced VvRab proteins with the 57 encoded AtRab proteins was used as a bootstrap analysis to construct an unrooted consensus phylogenetic tree (Fig. 2). Examination of the resulting tree indicated that the VvRabs, can be grouped into eight subfamilies, as for the Arabidopsis Rab family (Pereira-Leal and Seabra, 2001). More than half of the VvRabs belong to the RabA group, in six distinct subtypes (1–6). The other remaining 12 VvRab gene products can be divided into seven other groups, with three RabB members, three RabF members, two RabE members, one RabC, one RabD, one RabG, and one RabH subfamilies. Compared to Arabidopsis, grapevine generally has fewer homologous members in each of the eight subfamilies. The VvRabs were named according to their sequence similarity to Rabs from A. thaliana (AtRabs).

VvRab gene structure

The VvRab gene molecular organization was evaluated using the data obtained from the shotgun grapevine genome sequencing project (http://www.cns.fr/externe/Francais/Projets/Projet_ML/projet.html#biblio). The selected grapevine genomic sequences were annotated to determine the intron/exon structure of the VvRab genes (Fig. 3). Analysis of the intron–exon junctions deduced from alignment with the VvRab cDNAs sequences revealed that each of the Rab branches in grapevine have generally conserved introns/exon boundaries. All the Rab from the A subfamily have two exons, but the splice exhibited a different position according to the subtype. Groups B, C, and H have six exons, G has seven exons, D and E have eight exons, and group F exhibited various exon numbers (5, 7, and 8).

Localization of the VvRab genes on the grapevine genome

Eighteen of the 26 members of the VvRab gene family could be located on identified grapevine chromosomes (Fig. 4). Generally one VvRab was located per chromosome, but in some cases, two or three VvRabs were anchored on the same chromosome. For instance, VvRabA1c and VvRabA1f were both found on chromosome 10, between the UMC8A4 and UDV-063 markers and the VRZAG25 and VMC2A10 markers, respectively. It is the same for VvRabA4d and VvRabF1 that were both on chromosome 13, between the VMC3B12 and VMCN91D12 markers and the VVIH54 and VVIM63 markers, respectively. Finally, VvRabA4d1, VvRabE1a, and VvRabE1c were all found on chromosome 8, between VMC2H10 and the end of the chromosome 8, between the VMC9F4 and VMC1B11 markers, and between the A47D and VMCN92B6 markers, respectively. For the other VvRabs belonging to the same A subfamily, location on different chromosomes was observed: VvRabA1b, VvRabA1e, VvRabA2b, VvRabA3, VvRabA5b, and VvRabA6a were found to be located on chromosomes 19, 12, 6, 15, 5, and 17, between the VMC5H11 and VVIU09, VMC4A9 and VMC8G6, VDV-085 and VVIP72, VMC8G3-2 and VMC4D9-2, VMC6E10 and VVC71, and VMC3C11-VVSCU06 markers, respectively. Finally, VvRabB1b, VvRabC1, VvRabH1b, VvRabF2b1, and VvRabF2b2 were respectively placed on chromosomes 14, 18, 2, 9, and 11, between the A010 and A27E, VVINV3 and VCM2A7, VMC5G7 and VMC2C10-1, UVD132 and VMC3G8-2, and VMC6C3 and VVSV2 markers. The other VvRabs belong to the yet unassigned portions of the genome clustered in the ChrUn Random chromosome.
Grapevine organ profiling of VvRab transcripts

To elucidate the physiological functions of different VvRabs, 11 were selected, representing almost every group present in grapevine, and the constitutive expression levels of their transcripts were analysed in different grapevine organs: RNA samples from roots, buds, young expanding leaves, fully developed leaves, tendrils, inflorescences, stamens, pistils, flowers, and pips were...
Fig. 1. Multiple alignment of the *V. vinifera* Rab deduced amino acid sequences and consensus. Distinct functional domains are designed according to Pereira-Leal and Seabra (2000, 2001): Rab specific regions (F), Rab subfamily specific regions (SF), GDP/GTP-binding domains (G), phosphate/Mg²⁺ binding domains (PM), and the geranylgeranylation region (C).
amplified using real-time quantitative RT-PCR with sense and reverse 3' UTR gene-specific primers. The results were normalized using the expression level of the constitutive elongation factor EF1-α. Analyses showed that VvRab genes were expressed in all the grapevine organs investigated (Fig. 5), except for VvRabA5b. Among the most typical patterns, VvRabA2a transcripts were 5–20-fold more abundant in roots than in all other organs. VvRabE1c transcript levels generally exhibited the same expression level in all the organs investigated, except at a lower level in pips. For the other VvRabs, differing expression patterns were observed, even for genes belonging to the same group.

### Grape berry developmental profiling of VvRab transcripts

We were interested in determining whether VvRabs were expressed during the development of grape berries, and if so, whether the different genes were expressed in distinct patterns. No expression of VvRabA5b was detected along berry development, but all the 10 remaining VvRabs were strongly expressed in grape berries, exhibiting various expression patterns (Fig. 6). For each VvRab, transcript expression was observed at all berry development stages. Identical results were observed in a repeated experiment. For VvRabA1c, VvRabA1e, VvRabG3a, expression tended to decrease after the onset of ripening, i.e. véraison. For all other Rabs, expression pattern fluctuated (VvRabA2a, VvRabB1d, and VvRabD2c), or remained almost constant (VvRabA5e, VvRabB1c, VvRabCl, VvRabE1c) during berry development.

### Effect of treatments on grapevine suspension cells

VvRab expression was also examined by real-time quantitative RT-PCR in grapevine suspension cell cultures before and 24 h after different treatments with growth regulators. Treatments of grapevine cells with MCP, CEPA, ABA, or NAA led to various responses according to the VvRabs (Fig. 7). CEPA treatment led to a significant reduction of VvRabA5e and VvRabB1d expression, suggesting a down-regulation by ethylene. Correspondingly, this effect was not observed when cells were treated with MCP. ABA treatments significantly enhanced (VvRabA1c, VvRabA1e, VvRabA2a, and VvRabD2c) or decreased (VvRabB1d) transcript levels. VvRabA1c, VvRabA5e, VvRabB1c, and VvRabCl transcript levels were also reduced significantly in cells treated with NAA. No significant effect was observed for the other conditions.

### Profiling of VvRab proteins during grape berry development and in grapevine organs and tissues

Using a specific monoclonal antibody raised against Arabidopsis AtRabA5c (ARA4) GTPase (Ueda et al., 1996), western blot analyses of a series of extracts of developing berries and from grapevine organs and tissues revealed a single band (approximately 24 kDa) corresponding to the Rabs molecular mass (Fig. 8). A VvRab protein, very closely related to AtRabA5c, was found to be expressed mainly in the last stages of fruit development, i.e. during ripening, whereas no expression was detected before and at véraison (Fig. 8A). Interestingly, expression both in Cabernet Sauvignon and Alicante was limited to the exocarp, and no VvRab protein was detected in the mesocarp or in the other organs investigated (Fig. 8B, C). A repeated experiment (data not shown) specifically confirmed this presence only in the exocarp, in the red or white berry varieties tested.

### Discussion

In this paper, 26 VvRabs were identified, with the five Rab-specific motifs named F1 to F5, with the conserved PM/G motif, and, generally, a double cysteine C-terminal prenylation motif corresponding to the definition of a Rab GTPase (Pereira-Leal and Seabra, 2000). This number represents as exhaustively as possible the number of Rab genes in V. vinifera as deduced from the complete genome sequence (Jaillon et al., 2007). It is, however, smaller than the 57 Rab GTPases found in Arabidopsis, 51 in rice, and 41 in maize (Zhang et al., 2007). The nomenclature used in this work is that defined in Pereira-Leal and Seabra (2001). The comparison of the VvRab gene structure with the known Rab structures in Arabidopsis (Rutherford and...
Fig. 2. Bootstrap consensus tree from the parsimony analysis showing the phylogenetic relationship among the twenty six V. vinifera Rab sequences (VvRab), and the 57 A. thaliana Rab sequences (AtRab). The eight subfamilies identified previously for AtRab sequences are shown. GenBank accessions numbers for the Arabidopsis sequences are the same as described in Pereira-Leal and Seabra (2001).
Moore, 2002) revealed an almost complete conservation of the gene exon/intron structure and the same number of exons within each group of Rab genes. The VvRab genes could all be placed in the different phylogenetic groups of the Rab plant subfamily, and were numbered in accordance with their similarities to the A. thaliana Rabs. VvRabs from group A displayed two exons, those from groups B, C, and H six exons, that from group G seven exons, and those from groups D and E eight exons. However, contrary to Arabidopsis (Rutherford and Moore, 2002), the number of exons from group F is variable (5, 7, or 8). All identified VvRabs shared the typical, perfectly conserved domains involved in guanine and phosphate/Mg\(^{2+}\) binding (PM1 to PM3 and G1 to G3), and generally the double-cysteine motif in the C terminus (Pereira-Leal and Seabra, 2000). Analysis of the aligned sequences confirmed the positions of the five conserved short stretches of residues F1 to F5 described as Rab-specific sequences by Pereira-Leal and Seabra (2000), and also of the SF1 to SF3 subfamily-specific sequences. These data confirm the definition as Rab GTPases of the 26 sequences presented in this study. As for Arabidopsis, maize, and rice (Zhang et al., 2007), VvRabs from group A are predominant among all VvRab groups. It is interesting to note that VvRabF1 displayed some unique features, such as a lack at the C-terminal region of the hypervariable region and of the cysteine motif, and the presence of additional amino acids at the N-terminus. These features match those from AtRabF1 (ARA6)

![Molecular organization of the 26 V. vinifera Rab GTPases.](image)

![Genetic distribution of part of the V. vinifera Rab GTPase gene family.](image)
protein, which appear to be unique to plants (Ueda et al., 2001).

Eighteen out of the 26 VvRab genes could be quickly placed on the map of the *V. vinifera* genome, each generally locating on a different chromosome as expected from the ubiquitous repartition of small GTPases gene families in other species (Jiang and Ramachandran, 2006) and no evidence of any tandem clustering of these genes was found. We analysed, in the grapevine genome, the environment of the VvRab genes. Interestingly, it was found that two of them, VvRabA1e and VvRabA1f, located close (from 20–70 kb) to VvRops GTPases, and in a region enriched in sequences encoding for O-methyltransferase. For the other VvRabs, no obvious organization could be identified.

Possible functions of VvRabs were investigated through expression studies in various organs, during fruit development and in response to different cell treatments. As for other studies (Loraine et al., 1996; Lu et al., 2001), the present results showed that VvRabs were differentially regulated in different organs, but none of our data showed a single VvRab gene with expression restricted to a specific organ. VvRabA2a was mainly expressed in roots compared with other organs. For other VvRabs, various expression patterns were observed. In particular, expression was observed in roots for all the VvRabs analysed. This contrasts with the lack of expression observed by northern blotting in tomato roots for some Rabs from groups A and D (Loraine et al., 1996; Lu et al., 2001).

Gene expression for all 10 VvRabs was apparently not co-ordinated in the different tissues tested as well as during berry development. In fact, no single expression pattern could be observed during fruit development. These results contrast with northern blotting data showing an accumulation of the LeRab1A, LeRab1B (class D), and LeRab11a (subfamily A) mRNAs during tomato ripening (Loraine et al., 1996; Lu et al., 2001), of a Rab11 like
A gene in ripening mango fruit (Zainal et al., 1996) or a Rab7 (subfamily G) in ripening apricot (Mbeguei-A-Mbeguei et al., 1997). Phylogenetic grouping of VvRabs was not associated with a particular expression pattern or level in organs, during fruit development or in response to cell treatments. These results demonstrated that VvRab transcripts were generally constitutively and differentially expressed in grapevine, suggesting a constitutive and/or specific function of these genes throughout the entire plant lifetime. A 24 h-treatment with CEPA decreased VvRabA5e and VvRabB1d, whereas ABA treatment increased the transcript levels of VvRabA1c, VvRabA1e, VvRabA2a, and VvRabD2c and decreased that of VvRabB1d. In addition, NAA treatment decreased VvRabA1c, VvRabA5e, VvRabB1c, and VvRabC1. These data suggest an involvement of ethylene, abscisic acid, or auxin signalling in the control of VvRab transcript expression. Such involvements of ethylene and auxin have already been reported in other plant species (Moshkov et al., 2003a, b; Qi et al., 2005), with an up-regulation of the Rab transcript expression. This is also the case for abscisic acid, treatment with which in elongating cells of the embryonic axis from Fagus sylvaticus was shown to enhance expression of FsRab11a, a Rab from the A subfamily (Nicolas et al., 1998). Marked changes in the expression level of some VvRab genes after growth regulator treatment suggest that these VvRab proteins participate in cellular events triggered by plant hormones. Our results are consistent with a regulation of VvRab expression by hormonal signals, but regulation during fruit development is not obvious at transcript level. VvRab transcripts probably respond to distinct signalling pathways (as inferred from their various responses to plant growth regulators).

At the protein level, results of western blotting clearly indicated that a grapevine protein closely similar to

Fig. 6. Transcript profiling of V. vinifera Rab GTPases during Cabernet Sauvignon berry development. Total RNAs were isolated from 3–12 WPF berries. VvRab transcript levels were normalized to EF1-α transcript level used as endogenous reference (for correcting results from differing amounts of input RNA), and expressed as arbitrary units. Experiments were repeated twice (2002 and 2006); A: VvRabA1c, B: VvRabA1e, C: VvRabA2a, D: VvRabA5e, E: VvRabB1c, F: VvRabB1d, G: VvRabC1, H: VvRabD2c, I: VvRabE1c, J: VvRabG3a. Véraison is indicated by an arrow. Stars indicate the data not determined.
AtRabA5c (ARA4) was hybridized by the specific monoclonal antibody raised against AtRabA5c. This antibody seemed very specific, as shown in Anai et al. (1995). Contrasting with results from Ueda et al. (1996), a single 24 kDa product was detected by immunoblot using this antibody in developing berries and grapevine tissues. Expression of this VvRab protein from the A subfamily was developmentally-regulated, being strongly accumulated during the ripening stages. It was found that expression of this protein was specific to ripe berry tissues, and that no expression was detected in the other various grapevine organs tested. Moreover, expression of this protein was restricted to berry exocarp, independently of the colour of the variety analysed. To our knowledge, this is the first time that expression of a Rab protein is described as being specific of the exocarp of a ripe fruit, independently of the red or white berry varieties tested.

Comparison of VvRab protein sequences with the epitope sequence of AtRabA5c indicated that VvRabA5b and VvRabA5e, were the closest related proteins to the AtRabA5c one. In fact, these VvRabs displayed 72% identity to AtRabA5c, whereas the other VvRab proteins had less than 60%. At the transcript level, no VvRabA5b expression was observed in the different organs tested, or in developing berries (data not shown), whereas in contrast VvRabA5e transcripts were expressed in all the grapevine organs as well as during grape berry development. Thus, it is likely that the protein hybridized by western blotting is related to the VvRabA5e protein. Interestingly, VvRabA5e transcript expression level remained almost unchanged during fruit development, whereas VvRabA5e protein content was developmentally-regulated. These data could prove a strong post-transcriptional regulation of VvRabA5e. Such regulation has not been reported previously for any Rab in ripening fruits, although post-transcriptional regulation by auxin was reported (only in roots) for Rha1, an AtRabA5 homologue (Qi et al., 2005). In Arabidopsis, the endogenous RabA5c

Fig. 7. Transcript profiling of V. vinifera Rab GTPases in suspension cells from Cabernet Sauvignon before (white box) and after (black box) different treatments (MCP, 1-methylcyclopropene; CEPA, 2-chloroethyphosphonic acid; ABA, abscisic acid; NAA, α-naphthalene acetic acid). VvRab transcript levels were normalized to EF1-α transcript level used as endogenous reference (for correcting results from differing amounts of input RNA), and expressed as arbitrary units (Bars = SD). Experiments were repeated twice. (A) VvRabA1c, (B) VvRabA1e, (C) VvRabA2a, (D) VvRabA5e, (E) VvRabB1c, (F) VvRabB1d, (G) VvRabC1, (H) VvRabD2c, (I) VvRabE1c, (J) VvRabG3a.

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