Vitis **49** (4), 193–199 (2010)

Cellular localisation of VvRops and VvRabA5e, small GTPases developmentally regulated in grape berries

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Summary

VvRops, in particular VvRop9, and VvRabA5e are small GTPases which are developmentally regulated in grape berries. In an attempt to help elucidate the role of these proteins during fruit development and ripening, we investigated their localisation in the fruit by immunocytofluorescence. These proteins were observed at a perinuclear location, at cell periphery and around vesicles. In particular VvRops were found to be located in the nucleus and likely on the plasma membrane. VvRop9 and VvRabA5e cDNAs were introduced separately into *S. cerevisiae* mutants with *RHO1* and *YPT31/YPT32* defective genes respectively. Neither cDNAs could complement these temperature-sensitive mutants, suggesting that the functions of the VvRop9 and VvRabA5e genes in grapevine likely differ from the functions of *RHO1* and *YPT31/YPT32* genes in yeast.

K e y w o r d s : grape berry, immunocytofluorescence, Rab, Rop, small GTPases, yeast complementation.

Introduction

Small GTPases are ubiquitous monomeric protein switches which relay several signals in eukaryotic organisms (Ma 2007). In plants, this gene family is divided into four main subfamilies (Vernoud *et al*. 2003), among which Rops seem to be regulated in a unique, plant-specific manner (Berken 2006), and Rabs seem to respond to plant-specific signals (Ma 2007).

Rop family members play central roles in a wide range of cellular processes such as cell shape, polar cell expansion, plant-microbe interactions, plant development, phytohormone signalling and stress responses (Berken 2006, Gu *et al*. 2004, Molendijk *et al*. 2001, Yang 2002). Rop proteins regulate extracellular stimulus-dependent signalling pathways and likely associate with receptor-like kinases within high molecular weight complexes on the plasma membrane (Trotochaud *et al*. 1999, Wengier *et al*. 2003). In the *Vitis vinifera* genome, 7 VvRops cDNAs were found, and expression of these genes is consistent with a regulation by developmental and hormonal signals (Abbal *et al*. 2007). Among VvRops, VvRop9 displayed high berry specificity, a specific response to ABA, and distinct sequence elements suggesting characteristic protein-protein interactions, as well as a distinct subcellular localisation. Rab proteins are important signal transducers and essential elements that control nuclear and vesicular trafficking processes. They constitute, in Eucaryotes, the largest family of small monomeric GTPases and are ubiquitously expressed (Pereira-Leal and Seabra 2001). Different Rabs are localised on distinct vesicles and organelles, and the function of each Rab protein relates to its localisation (Chavrier and Goud 1999). Based on sequence homology, the 57 *Arabidopsis* Rabs (Pereira-Leal and Seabra 2001, Rutherford and Moore 2002) and the 26 *Vitis vinifera* Rabs (Abbal *et al*. 2008) have been grouped in eight functional families, one of which is the RabA subgroup. The *Vitis vinifera* genome has 10 VvRabs belonging to this subgroup. It has been shown that proteins belonging to one subgroup do not always display similar functions (Chavrier and Goud 1999), as in the case of the RabA family which presented a functional diversification in plants (Inaba *et al*. 2002). In grapevine, expression of VvRabA5e, a member of the RabA family, is not transcriptionally regulated during berry development, although it is thus regulated at protein level (Abbal *et al*. 2008). In fact, sequence similarity searches revealed that the possible orthologue of VvRabA5e protein in the *Arabidopsis* genome is AtRabA5c (ARA4). Interestingly, Western blotting performed using a monoclonal AtRabA5c antibody revealed a specific expression of a VvRabA5 protein in the exocarp of the ripening grape berries (Abbal *et al*. 2008).

The elucidation of the subcellular localisation of VvRops and VvRabs is essential to help elucidate their biological function. Thus as a first step, we determined the localisation in grape berries of VvRop and VvRab proteins by *in situ* immunocytology, using respectively polyclonal and monoclonal antibodies. Yeast complementation by VvRop9 and VvRabA5e was also performed to go further into the functional characterization of these two grapevine proteins.

Material and Methods

P l a n t m a t e r i a l : The plant material used in this study was from *Vitis vinifera* L. ('Chardonnay' and 'Shiraz') greenhouse-grown vines. Berries were harvested at 5 week-postflowering (WPF) for green developmental stage and at 10 WPF for ripening stage.

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Immunocytofluorescence technique for *in situ* VvRops and VvRabA5e localisation in sections of grape berries: Grape berry tissue preparation was performed mainly as described in Paciorek *et al.* (2006). The procedure involved a fixation step in a 10 mM, 7.2 pH phosphate buffered saline (PBS), containing 4 % paraformaldehyde, followed by step-wise dehydration in an ethanol series (from 50 % to 100 %) with a final 100 % butanol bath (5 d). Tissue embedding was performed with successive baths with increasing concentrations of Safesolv (Labonord, France) in butanol then with Safesolv/ Paraplast pure wax (paraffin X-TRA, McCormick Scientific) with increasing wax concentrations and at 45 °C to 58 °C. At this stage, each berry was embedded in wax at 4 °C in small molds and stored at 4 °C. Eight µm-thick sections were cut with a razor blade (Leica microtome RM 2265). Strips were placed on a warm water surface on a heating plate to unfold. Sections were transferred onto silanized-slides (DakoCytomation, Carpinteria, CA, USA) and completely dried. Samples were then dewaxed and rehydrated reversing the dehydration procedure.

Next, a blocking incubation with BSA in PBS (2 %) was performed, allowing to block non-specific protein binding. The BSA solution was removed thereafter and the primary antibody solution gently poured on the slides. We used a rabbit anti-AtRop polyclonal antibody and a mouse monoclonal anti-AtRabA5c (ARA4) antibody, respectively obtained from Dr. J. Bailey-Serres and Dr. T. Ueda, and previously used for Western blotting investigations in developing grape berries (Abbal *et al*. 2007 and 2008). After overnight incubation at 4 °C, sections were washed with PBS (3 x 10 min) and incubated in the secondary antibody (anti-rabbit or anti-mouse IgG $F(ab')_2$ fragment conjugated to the Alexa Fluor 488 fluorochrome (Molecular Probes, Invitrogen) for 1 h 30 at room temperature in darkness. The secondary antibody was then removed and after washing in PBS, a 2 µM DAPI solution (Sigma) was then pipeted onto the sections to specifically reveal the nuclei. After washing in water (three times), the sections were mounted in Mowiol antifading medium. Microscope imaging was performed at Montpellier RIO Imaging Platform (www.mri.cnrs.fr) with a multiphoton laser scanning Axiovert 200M 510 META NLO microscope (Carl Zeiss MicroImaging, Jena Germany). Excitation was provided by a Chameleon Ultra II Ti–sapphire laser (Coherent, San Jose, CA, USA) at 720 nm, an argon laser at 488 nm and a HeNe laser at 543 nm. We observed the sections with Plan Neofluar x 25/0.8 and C-Apochromat x 40/1.2 Zeiss objectives.

Pictures were processed using the Zeiss LSM image browser software (www.zeiss.com) and Photoshop CS3. Three-dimensional pictures and colocalisation analysis were obtained with Volocity 5.0.2 (Improvision, Perkin Elmer Company).

Yeast transformation: VvRop9 (EMBL accession no. A5ARL1) and VvRabA5e (EMBL accession no. A5B3S9) cDNAs were amplified from start to stop codons with gene-specific primers, containing a *Hpa*I restriction site (Tab. 1). Fragments were inserted in the *Hpa*I site of the yeast centromeric expression vector pMET189, an URA3 *Escherichia coli*-yeast shuttle plasmid, in which the met25 promoter and the CYC1 terminator flanked the *Hpa*I cloning site. These constructs, together with control plasmids, were introduced into the *Saccharomyces cerevisiae* strains listed in Tab. 2. Yeast cells were plated and grown on yeast peptone dextrose (YPD) medium (1 % bactoyeast extract, 2 % bactopeptone, 2 % glucose). Colonies were then cultured in 20 ml liquid YPD medium at 28 °C for one night and subcultured in the same medium. Transformation was performed using the lithium acetate procedure (Gietz and Woods 2002). For complementation testing, transformants were streaked onto synthetic medium YNB (0.67 %

Table 1

Primers 5'-end and 3'-end used for full length VvRop9 and VvRabA5e cDNAs from ATG to stop codon

Pair name	Primer 5'-end forward sequence	Primer 3'-end reverse sequence	Product length (bp)
VvRop9 F/R	NNNGTTAACATGAGCGCTTCGAAGTTC	NNNGTTAACTGCTGCAGCACAGCCTCC	630
VvRabA5e F/R	NNNCCCGGGATGGGGGAAGAAGGAGAG	NNNCCCGGGTCTGGAACAGCAAGAAGA	645

Table 2

Genotypes and phenotypes of the *S. cerevisiae* strains used in the present study

Mutant	Strain	Genotype	Phenotype
rho1.2	YOC772	*MAT**a** ade2 his3 leu2 lys2 trp1 ura3 rho1*::*HIS3 ade3*::[rho1-2 LEU2]	Heat sensitive (37 °C)
rho1.3	YOC773	*MAT**a** ade2 his3 leu2 lys2 trp1 ura3 rho1*::*HIS3 ade3*::[rho1-3 LEU2]	Heat sensitive (37 °C)
Control	YOC784	*MAT**a** ade2 his3 leu2 lys2 trp1 ura3 rho1*::*HIS3 ade3*::[RHO1 LEU2]	Normal
cdc42	YAT2297	*MATα ura3 cdc42-1*	Heat sensitive (37 °C)
Control	X2180	*MATα ura3*	Normal
ypt32	YTH11	*MAT**a** ypt32*::*HIS4 ura3 leu2 his4 lys2*	Cold sensitive (12 °C)
ypt31	YTH12	*MAT**a**ypt31E49Q-LEU2 ypt32*::*HIS4 ura3 leu2 his4 lys2*	Cold sensitive (12 °C)
Control	BY4742	*MATα ura3 leu2 his3 lys2*	Normal

Bacto yeast nitrogen base, 2 % glucose supplemented with appropriate amino acids but lacking uracil) and incubated for 3 d at 28 °C (permissive temperature). Colonies were then plated and cultured 3 d at 37 °C, the restrictive temperature for YOC772, YOC773 with YOC784 as control, also for 3 d at 37 °C for Cdc42ts with X2180 as control, and finally 10 d at 12 °C, the restrictive temperature for YTH11 and YTH12 with BY4742 as control.

Results

In situ localisation of VvRops and VvRabA5e in cells of grape berries: To begin assessing potential roles for Rops and Rabs in grape berries, we determined their tissular and subcellular localisation using an indirect immunofluorescence technique with anti-Rop and anti-RabA5c antibodies. Rop antibodies do not discriminate between the different Rop proteins. The search for the localisation of VvRops and VvRabA5e was performed on cross-sections of young or ripe berries embedded in Paraplast. Different checks were performed to evaluate the specificity of the immunofluorescence signal observed. Controls with no primary anti-bodies revealed the autofluorescence background of grape berry in the optical conditions of the experiment, *i.e.* a red auto-fluorescence of the cuticle and cell layers under the cuticle (Fig. 1 A and Fig. 2 A) or a yellow-green autofluorescence of the xylem vessels (Fig. 1D). The red autofluorescence observed in the cells of epicarp likely corresponded to phenolic compounds.

With a polyclonal anti-Rop antibody on young berries, a specific green fluorescence was found in all epicarp cells (Fig. 1 B and C) and in the vascular tissues mainly at the phloem level (Fig. 1 E and F), indicating the presence of VvRop proteins. The signal specificity was checked by spectral analysis using the Linear Unmixing method (Zeiss 510 Meta, data not shown).

Closer examination of epicarp cells revealed that the specific signal was found in the cytoplasm, around "vesicles" or spherical inclusions of phenolic compounds and around the vacuole (Fig. 1 C), but it was particularly dense around and inside the nucleus. In the phloem cells, the specific signal was found in the cytoplasm (Fig. 1 F). DAPI staining of the same samples showed that the nucleus could be superimposed on the green specific signal (Fig. 1 G). To confirm this nuclear localisation, we studied the spatial distribution in three-dimensional images obtained from

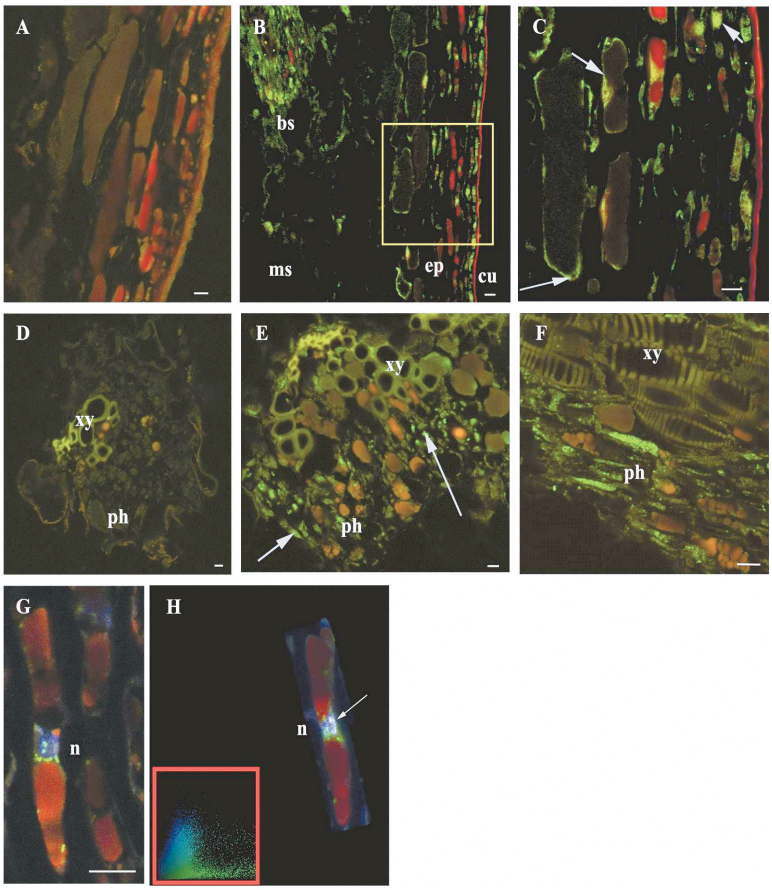

Fig. 1: Localisation of VvRops proteins in 'Chardonnay' green berries. (**A**, **D**) Controls with no primary antibody and with secondary anti-rabbit antibody display cells with background autofluorescence. (**B**, **C**, **E**, **F**, **G**) Immunofluorescent VvRops localisation (green, arrows) and DAPI labelling nuclei (blue). (**B**, **C**) VvRops localisation in epicarp (square in B localizing the magnified zone **C**) and (**E**, **F**) VvRops localisation in respectively transversal or longitudinal section bundle sheath. (**G**) Subcellular localisation in a cell of epicarp. (**H**) Visualization of fluorescence in 3D rendering from Z-sections (Volocity Visualization) and colocalisation (white pixels) with DAPI; insert: scatter blot between green channel and blue channel. bs : bundle sheath, cu : cuticle, ep : epicarp, ms: mesocarp, n : nucleus, ph : phloem, xy : xylem. Bar = 10 µm.

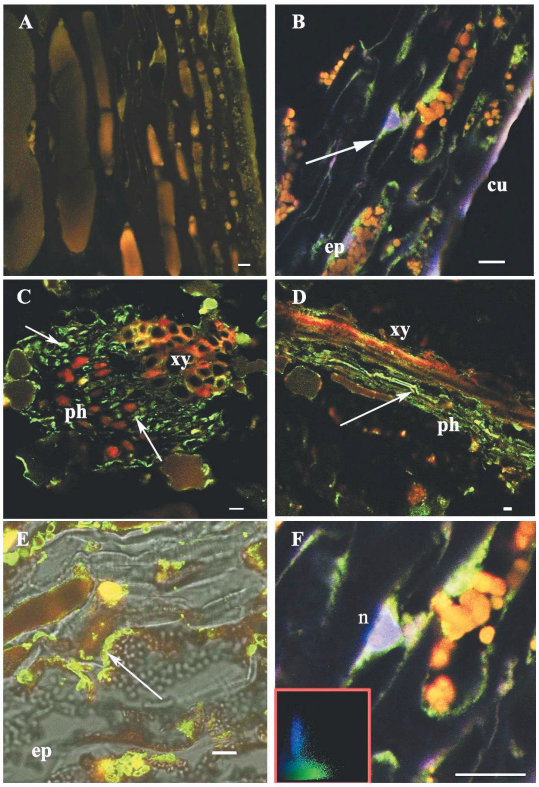

Fig. 2: Localisation of VvRabA5e protein in 'Shiraz' ripe berries. (**A**) Control with no primary antibody and with secondary anti-mouse antibody indicates cells with background autofluorescence. (**B**, **C**, **D**, **E**, **F**) Immunofluorescent VvRabA5e localisation (green, arrows) and DAPI labelling nuclei (blue). (**B**, **E**) VvRabA5e localisation in epicarp. (**C**, **D**) VvRabA5e localisation in respectively transversal or longitudinal section bundle sheath. (**F**) Subcellular localisation in epicarp cells; insert: scatter blot between green channel and blue channel shows no-colocalisation between green specific signal and DAPI. cu: cuticle, ep : epicarp, n : nucleus, ph : phloem, xy : xylem. Bar = 10 µm.

Z-stack acquisition (Fig. 1 H). Overlay methods allowed to visualize the colocalisation (superposition) between DAPI and Alexa-Fluor 488 within the sample (Fig. 1 H, white pixels, video S1 supplementary data); however to confirm the localisation of Rop protein inside the nucleus and not only around, we studied colocalisation by correlation analysis based on Pearson' coefficient on three-dimensional images. The scatter plot obtained with this analysis showed a partial colocalisation indicating that VvRop proteins were also present in the nucleus (Fig. 1 H insert).

A specific immunofluorescence was also detected at the phloem level indicating the presence of VvRop proteins in the area of the vascular tissues (Fig. 1 E and Fig. 1 F). In ripe berries a weaker fluorescence was observed compared to young berries. This fluorescence was mainly restricted to the nuclear region for all cell types (data not shown). Identical results were obtained from either 'Chardonnay' or 'Shiraz' berries.

When immunolocalisation with a monoclonal AtRabA5c antibody was performed on ripe berries, a green specific fluorescent signal indicating the presence of the VvRabA5e protein was observed in the epicarp cell layers (Fig. 2 B) and in the vascular tissues mainly at the phloem level (Fig. 2 C and Fig. 2 D). At subcellular level, the specific green signal was found in the cytoplasm as a reticular form around vesicles and around the nucleus (Fig. 2 B, Fig. 2 E and Fig. 2 F). In the phloem, the specific signal was found at the periphery of the cells (Fig. 2 D). The imaging after DAPI labelling did not reveal the presence of the VvRabA5e protein inside the nucleus (Fig. 2 F). Colocalisation analysis between DAPI and green specific showed a negative correlation: the VvRabA5e protein was not present in the nucleus (Fig. 2 F insert). When the same experiment was performed on young berries, the fluorescence localisation was similar to that in ripe berries but the signal was weaker (data not shown). Identical results were obtained from either 'Chardonnay' or 'Shiraz' berries.

Yeast mutant complementation by VvRop9 and VvRabA5e: To investigate whether VvRop9 and VvRabA5e can complement yeast mutants, VvRop9 and VvRabA5e coding sequences were placed under the control of the met25 promoter on a single-copy plasmid and introduced into yeast strain mutants and their respective controls, as shown in Tab. 2. The mutated strains used for VvRop9 and VvRabA5e display heat-sensitive (YOC772, YOC773 and YAT2297) and cold-sensitive phenotypes (YTH11 and YTH12), respectively. To study whether VvRop9 has a function similar to yeast *RHO1* and *CDC42*, *rho1.2*, *rho1.3, cdc42*-defective yeast strains and YOC784 or X2180 control strains were transformed with the pMET189 plasmid containing or not the VvRop9 cDNA. Transformants were grown on YNB medium for 3 d, either at 28 °C or at 37 °C. At 28 °C, growth was similar between the VvRop9 transformed YAT2297 (*cdc42*) and the X2180 control (Fig. 3 A), whereas at 37 °C, only the control strain grew (Fig. 3 B). The same results were obtained with transformed YOC772 (*rho1.2*) and YOC773 (*rho1.3*) strains and their related YOC784 control strain (data not shown). These results suggest that VvRop9 cannot complement *rho1.2*, *rho1.3* and *cdc42* yeast mutants.

Concerning the VvRabA5e function, YTH11 (*ypt32*) and YTH12 (*ypt31*) strains were transformed with the pMET189 plasmid containing or not VvRabA5e cDNA and compared to the BY4742 control strain. After 3 d at 28 °C, a

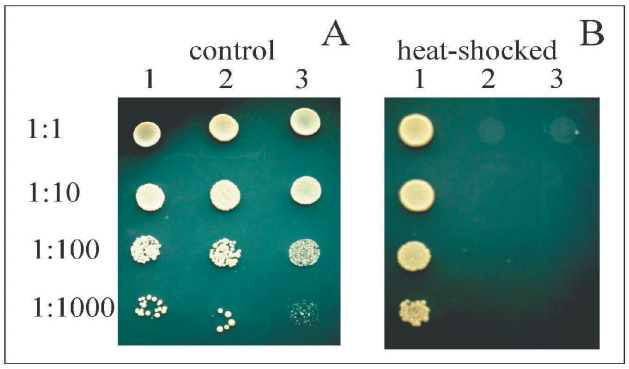

Fig. 3: Yeast mutant complementation with the VvRop9 cDNA. Serial dilution (1:1 to 1:1000, up to down) of yeast cells spotted onto YNB plate and incubated (**A**) at 28 °C (control) or (**B**) at 37 °C (heat shocked) for 3 d: X2180 control strain (1), cdc42 mutant strain containing plasmid without (2) or with (3) VvRop9 cDNA. The experiment was repeated with similar results.

similar growth was observed between transformed YTH12 (*ypt31*) mutants and the BY4742 control (Fig. 4 A). After 10 days at a restrictive temperature of 12 °C, the transformed mutant strain exhibited a strong growing limitation compared to the control (Fig. 4 B). Similar results were obtained with the YTH11 strain (data not shown). These data indicated that the cDNA encoded by VvRabA5e did not functionally complement the yeast mutant with cold sensitivity phenotype.

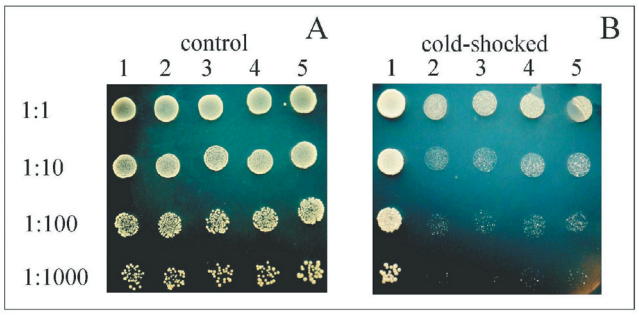

Fig. 4: Yeast mutant complementation with the VvRabA5e cDNA. Serial dilution (1:1 to 1:1000, up to down) of yeast cells spotted onto YNB plate and incubated (**A**) at 28 °C for 3 d (control) or (**B**) at 12 °C for 10 d (cold shocked): BY4742 control strain (1), YTH12 mutant strain containing plasmid without (2) or with (3 to 5) VvRabA5e cDNA. Lines 3 to 5 correspond to independent clones. The experiment was repeated with similar results.

Discussion

In previous works it has been shown that grapevine VvRops, in particular VvRop9, and VvRabA5e were developmentally regulated in grape berries (Abbal *et al*. 2007 and 2008). VvRop9 and the other VvRops were expressed at both transcript and protein levels in young berries, while VvRabA5e was specifically expressed at the protein level in ripening berries. The specificity of these expression profiles suggests coordinated roles for these small GTPases in berries, even though the functions of these proteins in relation to fruit development and ripening remains unknown. The present study aims at gaining more insight on tissue expression and subcellular localisation of VvRops and VvRabA5e that could provide clues to their functions in grape berries. As the recruitment of small GTPases to a specific subcellular membrane is crucial for their proper functions, we thus analysed the localisation of these small GTPases within grape berry cells. The data obtained in this study rely on using *in situ* immunocytofluorescence techniques.

Immunocytofluorescence techniques, using polyclonal anti-AtRops antibodies, revealed that VvRop proteins were present in distinct locations in young berries. At tissue level, localisation was observed in epicarp and in vascular tissues. Thus, these data confirm the observation made previously with the Western blotting approach, *i.e.* presence of VvRops in the pericarp of young berries (Abbal *et al*. 2007). In contrast, visualization of the mesocarp cells was difficult. In fact, these cells possess a very large vacuole which renders a good morphological conservation difficult when using wax embedding.

At subcellular level, VvRops localised at cell periphery, around the small vesicles, around and inside the nucleus, and also around the vascular tissues at phloem peripherical ring. A colocalisation of the VvRops immunocytofluorescence and the DAPI staining was revealed. This was statistically confirmed with Pearson's coefficient determination. However, this does not imply a direct interaction between VvRops and nuclear DNA. To our knowledge, this is the first report on Rops presence of in plant nucleus revealed by immunocytofluorescence.

Localisation of Rop GTPases in vascular regions has been reported in several papers: in *Zinnia elegans*, Rop GTPase ZeRAC2 mRNAs accumulated speciallly in xylem parenchyma, tracheary element precursor of mesophyl cells, and in phloem cells (Nakanomyo *et al*. 2002); during wood formation in Eucalyptus, some components of the Rop GTPase signalling were among the genes expressed preferentially in xylem cells (Paux *et al*. 2004); in *Arabidopsis* also, AtRop7, which was identified as part of the gene network regulating secondary xylem development (Ko *et al*. 2006), was found to be specifically expressed during late stages of xylem differentiation (Brembu *et al*. 2005). Thus, as it has been suggested for other tissues, VvRops could be involved in the differentiation of vascular tissues in the skin area of grape berries.

Different subcellular localisations have been described for Rops in the literature: perinuclear organelles, plasma membrane, tonoplast of developing vacuoles (Li *et al*. 1999, Lin *et al.* 2001; Fu *et al*. 2001). For instance, in *Picea willsonii* pollen tubes, indirect immunofluorescence microscopy revealed that Rac1 GTPase localised to specific regions of the plasma membrane (Zhang *et al*. 2007). Moreover, fluorescence from the anti-NtRac1 antiserum was observed around the nucleus in growing tobacco pollen tubes. These data suggest a potential function of Rac1 in nucleus signalling. In pea anthers, anti-Rop-linked immunofluorescence was observed as a circle aligning just outside the nucleus (Lin *et al.* 2001). In *Arabidopsis* also, Bischoff *et al.* (2001) have observed the localisation of the GFP-AtRop4 fusion protein in the perinuclear region. For Rops of the same group II as VvRop9, plasma membrane, perinuclear and nuclear localisations have been reported. For instance the GFP-NtRac1 fusion protein (91 % identity between NtRac1 and VvRop9) was localised onto the cell membrane (Tao *et al*. 2002). Interestingly, transformation of protoplasts with a tobacco Rac-GDI resulted in a decrease of the association of GFP-NtRac1 with the cell membrane, with a corresponding increasing nuclear and perinuclear localisation. For OsRac1, (84 % identity between OsRac1 and VvRop9), a plasma membrane localisation was reported (Ono *et al*. 2001). This strong homology between VvRop9 and other Rops from group II suggests that the plasma membrane could be another localisation of VvRop9. In fact, analysis of the grapevine plasma membrane proteome revealed the presence of a protein closely related to VvRops in grape berries (Zhang *et al*. 2001). This unknown plasma membrane protein is 84 % identical to VvRop9, which represented the best match among all VvRops (J. Zhang personal communication).

As part of our trial to understand the function of VvRop9, we investigated whether VvRop9 could complement the *rho1.1*, *rho1.2* or *cdc42* yeasts mutants. The yeast *S. cerevisiae RHO1* gene, which is a homologue of the mammalian RhoA gene, is essential for cell viability (Yamochi *et al*. 1994). The *S. cerevisiae CDC42* gene product is involved in the morphogenetic events of the cell division cycle (Johnson and Pringle 1990). In the present study, complementation results revealed that the protein encoded by the VvRop9 cDNA did not functionally complement any of these mutant yeast phenotypes, *rho1.1*, *rho1.2* or *cdc42*. These data suggest that VvRop9 may be regulated by a signal pathway differing from that in *S. cerevisiae* or may have unique signalling roles in plants irrelevant to the function of yeast RHO1 or CDC42.

The immunocytofluorescence techniques with monoclonal AtRabA5c antibody revealed a similar localisation in the epicarp and in the phloem of VvRabA5e in ripening berries than VvRops in young berries. These data confirm the observation previously obtained with the Western blotting technique, *i.e.* presence of VvRabA5e proteins in pericarp of ripe berries (Abbal *et al*. 2008). At subcellular level, three localisations were revealed by fluorescence in the present study: at the border of the cells, around the vesicles and in the perinuclear zone. However, no fluorescence was detected inside the nucleus. In the literature (less abundant for plant Rabs than for Rops), different localisations have been reported. For instance, AtRabA5c (ARA4) from *Arabidopsis*, which is closely related to VvRabA5e, was localised at the Golgi cisterna and in vesicles around the Golgi, thus suggesting that its function is related to the Golgi apparatus function (Anai *et al.* 1995, Ueda *et al*. 1996). Analysis of *Arabidopsis* proteome revealed that some Rab proteins, among which RabA related proteins, localised at the plasma membrane (Marmagne *et al*. 2004). These results highlight a role for members of the RabA GTPase family in trafficking events between trans-Golgi network and plasma membrane (Nielsen *et al*. 2008), but no specific function could be assigned to the AtRabA5c protein. In grapevine, none of the spots identified in berry plasma membrane proteome corresponded to VvRab proteins (Zhang *et al*. 2008).

Several groups have reported that higher plants Rabs could complement *ypt* mutations of budding yeast, indicating an evolutionary conservation of this gene family (Kim *et al.* 1996). However, this is not a general case (Eom *et al*. 2006): in *Pisum sativum* and *Arabidopsis*, expression of respectively PRA2 and ARA4 proteins (both RabA-related) aggravates the growth defect of the mutants (Matsuda *et al*. 2000, Ueda *et al.* 1996). In the present study we have shown that VvRabA5e protein was unable to complement *ypt31* and *ypt32* mutants. In yeast, *YPT31/YPT32* regulate exit of secretory and endocytic cargo from the trans-Golgi cisterna (Chen *et al.* 2005), and play also a role in cell cytokinesis (Ortiz and Novick 2006). Thus, the function of VvRabA5e in grapevine is likely irrelevant to that of yeast *YPT31* or *YPT32*. The non-complementation of yeast mutants did not allow going further into the understanding of the function of the VvRop9 and VvRabA5e proteins, except that their functions diverge from that of RHO1 and CDC42 for VvRop9 and that of YYP31 and YPT32 for VvRabA5e.

To our knowledge, this is the first report on the subcellular localisation of Rops and Rab GTPases in a fruit at different developmental stages. Cellular localisation, immunofluorescence and confocal microscopy revealed that in grape berries, VvRops and VvRabA5e were predominantly localised in the cells of the skin and around the vascular tissues. From a subcellular point of view, a specific fluorescent signal was observed in the cytoplasm, around vesicles and nucleus for VvRops and VvRabA5e, and inside the nucleus only for VvRops. These data might point to a potential function of these proteins in signalling mechanisms in relation with vesicle trafficking within the cell of the grape berries. It is likely that vesicular traffic plays an important role in fruit-specific phenomena such as ripening, although how it is achieved remains completely unknown. This is an open question that will probably be clarified in the next future.

Acknowledgements

We are grateful to Dr. T. Ueda for monoclonal anti-AtRabA5c antibody and for yeast strains, to Dr. J. Bailey-Serres for the polyclonal anti-AtRop antibody and to Pr. F. Doignon for yeast strains. We also thank Dr. P. Chatelet for critical reading of the manuscript, and G. Albagnac for supporting our research effort.

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Received March 26, 2010