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Analysis of the transcript levels of *VvAdh1*, *VvAdh2* and *VvGrip4*, three genes highly expressed during *Vitis vinifera* L. berry development

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Summary

Well defined promoter sequences are required for the targeted expression of genes in transgenic grapevines. This paper describes a detailed study of the expression of three genes with potentially useful promoters. We have used real-time RT-PCR to evaluate the transcript levels of *VvAdh1*, *VvAdh2* and *VvGrip4* genes in various tissues (root, bud, tendril, inflorescence, fruit and embryogenic callus) of *Vitis vinifera* L. at different stages of development. Transcript levels of the three genes were highest in berry tissues but each had a distinct pattern of expression. *VvAdh1* showed higher transcript levels during the early stages of berry development, while levels of *VvAdh2* and *VvGrip4* were higher during ripening. However, none of these genes was expressed in a strictly fruit-specific manner. In particular, significant levels of *VvAdh1* and *VvGrip4* transcripts were observed during late tendril development and in the inflorescence, respectively. Transcript levels of all three genes were similar in both flesh and skin, indicating no tissue-specificity within the berry. Promoter sequences of the *VvAdh1*, *VvAdh2* and *VvGrip4* genes could be very useful to drive ectopic gene expression in berries of transgenic plants.

K e y w o r d s : alcohol dehydrogenase; gene expression; Grip; fruit development, promoter; *Vitis vinifera*.

Introduction

Grape berry development is characterized by a number of distinct phases. These include fruit set, the first (preveraison or stage I) phase of berry expansion during which cell division and expansion occur, the lag phase (stage II) when berry growth is slowed-down or halted and the second (postveraison or stage III) cell (and berry) expansion phase during which ripening takes place. Recent studies (*e.g.* Davies and Robinson 2000, Goes da Silva *et al.* 2005, Terrier *et al.* 2005) have identified a range of genes which are differentially expressed during the various stages of development. Such differentially expressed genes provide considerable scope for identifying genes with promoters that will be useful for driving ectopic gene expression in transgenic grapevines. Currently most transgenes in grapevine are expressed by the Cauliflower Mosaic Virus 35S promoter (Tesniere *et al.* 2006) but more targeted transgene expression systems allowing control over transcript level and temporal and spatial accumulation will be of use in a number of ways.

Of the genes that exhibit large changes in steady state transcript levels during berry development some, such as *VvAdh1*, are mainly expressed during the pre-veraison phase, while others such as *VvAdh2* and *VvGrip4* are expressed during ripening (Davies and Robinson 2000, Tesniere and Verries 2000).

Alcohol dehydrogenase (*Adh*) gene expression has been widely studied in various plants. Many *Adh* genes are stress-induced, particularly by low-oxygen environments (Gerlach *et al.* 1982, Gregerson *et al.* 1991, Dolferus *et al.* 1994, Millar *et al.* 1994). *Adh* genes have also been implicated in developmental events such as anther development and fruit ripening (Bucher *et al.* 1995, Gregerson *et al.* 1993, Ingersoll *et al*. 1994, Kyozuka *et al.* 1994, Tadege *et al.* 1997). In addition, members of *Adh* gene families are differentially expressed in different tissues. For example, in tomato, where three *Adh* genes have been characterized, *Adh1* is expressed in dry and germinating seed and in pollen (Tanksley 1979, Bicsak *et al.* 1982), *Adh2* is found in developing seed, ripening fruit and in cultured cells (Chen and Chase 1993), whereas *Adh3* is expressed in anthers (Ingersoll *et al.* 1994). In grapevine, *Adh* transcript accumulation was investigated in several tissues (Sarni-Manchado *et al.* 1997, Tesniere and Verries 2001) by northern blotting with an homologous *VvAdh* probe. No signal was detected in unstressed roots, but very low levels of transcripts were detected in young leaves and young berries. Tesniere and Verries (2000) detected transcripts of three isogenes in young berries, with *VvAdh2* representing the major isogene expressed in ripening fruit.

Davies and Robinson (2000) reported a number of grape ripening induced (Grip) genes whose transcript levels increased greatly at veraison. One of these, *VvGrip4*, matches most closely to an hydroxyproline-rich protein from sunflower and may be involved in cell wall structure. *VvGrip4* transcripts were detected by northern blotting at high levels in ripening grapes but were not detected in roots and leaves and therefore *VvGrip4* was suggested to have a function specific to berry ripening.

To gain further insight into the specificity of *VvAdh1*, *VvAdh2* and *VvGrip4*, expression, tissues from various developmental stages of a range of grapevine organs were

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analyzed using the sensitive and isogene specific real-time RT-PCR method. This paper presents new data on the transcript accumulation of three genes highly expressed during grape berry development.

Material and Methods

Grapevine tissues for transcript accumulation analysis: To investigate the temporal and developmental accumulation of the grapevine *VvAdh1*, *VvAdh2* and *VvGrip4* transcripts in different tissues and during fruit development, total RNA was isolated from vegetative organs, a berry developmental series from inflorescence to ripe berries and embryogenic tissues at different stages.

The vegetative organs analyzed were from *Vitis vinifera* L. cv. Cabernet Sauvignon vines and included young roots, shoot tips (including 2-3 expanded leaves), young leaves, tendrils (at 4 stages of development from very young organs taken from opening buds to mature tendrils) and buds (samplings 4-12, green buds taken from flowering axis; samplings 5-8, latent buds taken during winter 1 month before bud burst).

For berry development analysis, RNAs were isolated from *V. vinifera* cv. Cabernet Sauvignon inflorescences (stage 7, immature inflorescence emerging from buds; stage 17, mature inflorescence with all flowers formed and separated; F, flowers at flowering time, stages as defined by Coombe (1995) and seedless berries from 2 weeks post flowering to 16 weeks post flowering. RNAs from *V. vinifera* cv. Shiraz skin or flesh from flowering to 16 weeks post flowering were also included.

In vitro-grown *V. vinifera* cv. Portan embryogenic tissues corresponding to lightly differentiated callus with undifferentiated embryogenic masses (stage L) or highly differentiated callus with globular to advanced torpedo embryo-like structures were also analysed (stage H).

RNA isolation and cDNA synthesis: Total RNA from vegetative organs, flowers and berries was extracted from 1-2 g of ground, frozen tissues according to Rezaian and Krake (1987) with slight modifications as described by Boss *et al.* (2001). One hundred µg of crude RNA was purified using the Qiagen RNeasy Mini Protocol cleanup procedure (Qiagen, Hilden, Germany), including a DNase treatment, resulting in the isolation of 10-30 µg of clean RNA. Purified RNA from embryogenic callus was extracted from 100 mg of tissue using a Qiagen RNeasy kit. After a denaturation step (5 min, 95 °C), the quality and quantity of RNA was checked on a 1 % agarose gel and by spectroscopy using a nanospectofluorometer (ND-1000, NanoDrop Technologies, Rockland, USA). First strand cDNA was synthesised from 1 µg of purified RNA using an oligo(dT)20 primer with a SuperscriptIII RNase H- RT kit (Invitrogen, Carlsbad CA, USA) according to the manufacturers instructions but omitting the recombinant RNase inhibitor addition. After 1 h incubation at 50 °C the reaction was stopped by heating at 70 °C for 15 min and the cDNA diluted 1:10 to 1:20, as PCR with undiluted cDNAs tended to contribute to non specific amplification.

Real-time PCR analysis: Using Primer3 software (Rozen and Skaletsky 2000), highly specific primers (Tm = 58 °C) were designed to amplify 100-200 bp fragments from different regions of the 3‘ end of the ORF or in the 3’ UTR of the following genes; *VvAdh1* (GB AF194173), *VvAdh2* (GB AF194174), *VvGrip4* (GB AJ237982) and *VvUbiq* (GB CF406001).

Each PCR reaction, of 15 µl final volume, contained 5 µl of template cDNA, 333 nM of each primer and 1X SYBR® Green PCR master mix (Applied Biosystems, Warrington, UK). Thermocycling conditions were as follows: an initial enzyme activation of 10 min at 95 °C, was followed by 35-50 cycles of denaturation for 20 s at 94 °C, annealing for 20 s at 58 °C, and extension for 20 s at 72 °C, followed by a final extension step of 5 min at 72 °C and a melt gradient starting from 50 °C and heating to 96 °C at a rate of 0.2 °C s^{-1}. The real-time PCR reactions were carried out in a Rotor Gene™ 3000 Real Time PCR instrument (Corbett Research, Australia). The fluorescence of reactions was measured with an excitation wavelength of 470 nm and a detection wavelength of 510 nm at the end of each extension step and at each 1 °C increment of the melt profile.

All cDNA samples to be compared for transcript levels were analyzed in triplicate in a single batch for each primer pair for each gene. 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 make template standards. The most concentrated standard was assigned an arbitrary transcript copy number and the subsequent serially diluted standards were assigned relative copy numbers according to the fold dilution. Standards from 10^{-5} to 10^{-9} of the gene to be analyzed and from ubiquitin were included in the real-time PCR assay of cDNA samples. In each case, 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. Sample values were corrected using the corresponding expression level of ubiquitin and expressed as the average +/- standard deviation. Specificity of the PCR product generated for each set of primers was tested in three ways: (1) by the melt gradient in which fluorescence decreases at a single discrete temperature indicating separation of the two strands of a single DNA species, (2) by the detection of a single PCR product of the correct size on an agarose gel and if the previous test was not clear, (3) by cloning the product in pGEM-T easy (Promega, Madison, WI, USA) and sequencing.

Results

VvAdh1 transcript accumulation in various tissues: Transcript levels of *VvAdh1* (Fig. 1 A) were relatively high during stage I of Cabernet Sauvignon berry development, with the maximum level being observed 4 weeks after flowering. After veraison, the transcript level was considerably lower but still detect-

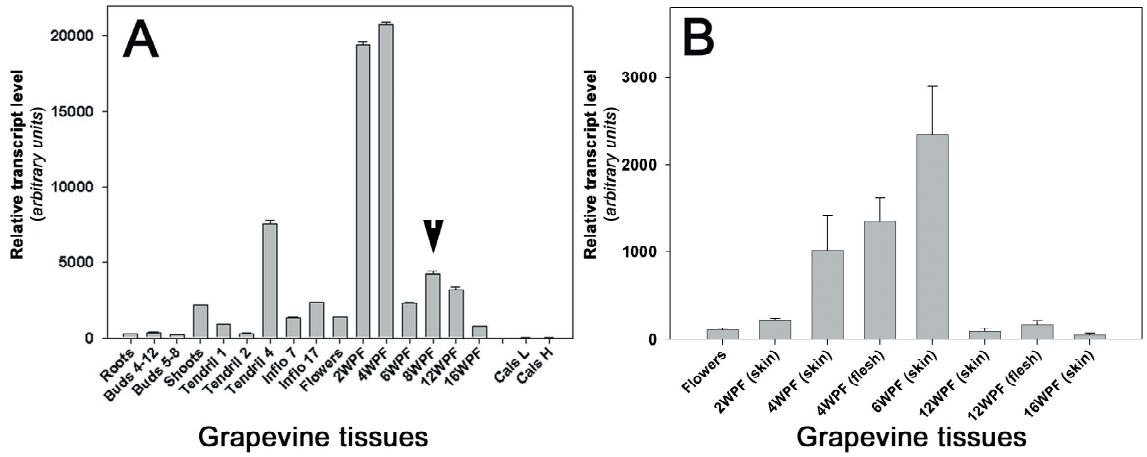

Fig. 1: Quantitative real-time PCR analysis of *VvAdh1* gene transcript accumulation in (**A**) various Cabernet Sauvignon grapevine tissues at different stages of development and undifferentiated (stage L) and highly differentiated (stage H) embryogenic callus of the cultivar Portan and (**B**) in skin and flesh tissues of Shiraz grape berries (Bars = SD). Transcript levels are expressed as arbitrary units relative to Ubiquitin. Veraison is indicated by an arrow. WPF: week-post-flowering.

able. Transcripts were also found in shoots and in mature tendrils as well as in inflorescence at all stages of development. The transcript levels in embryogenic callus were very low. A similar pattern was observed in Shiraz berries with high transcript levels during stage I of berry development and low levels during stage III (Fig. 1 B).

When transcript accumulation in separated skin and flesh tissues was compared (Fig. 1 B), *VvAdh1* was found to be expressed at similar levels in both indicating a lack of tissue specificity of expression within the berry. It should be noted that because of their relatively small volumes, especially later in berry development, the contribution of skin and seed to the total berry volume are considerably smaller than flesh.

VvAdh2 transcript levels in various tissues: *VvAdh2* (Fig. 2 A) transcript levels were relatively high in ripening berries, compared to other tissues, particularly during the later stages of development. However, transcripts were also observed in young berries just after flowering. Transcript levels in all other organs and in embryogenic callus were found to be very low. *VvAdh2* gene transcripts were detected at similar levels in separated Shiraz skin and flesh, indicating, as seen for *VvAdh1*, a lack of tissue-specific expression in the berry (Fig. 2 B).

VvGrip4 transcript levels in various tissues: Compared to *VvAdh1* or *VvAdh2*, *VvGrip4* (Fig. 3 A) exhibited the highest level of transcript accumulation, fluorescence levels increased significantly from 10 cycles even after using highly diluted cDNA templates. During berry development, *VvGrip4* showed a similar profile of transcript accumulation as *VvAdh2*, exhibiting the highest level of transcript accumulation at the later stages of development. However, the dramatic increase that occurs 8 week-post-flowering (wpf) (*i.e.* just at the onset of ripening) for *VvGrip4* transcripts (Fig. 3 A) occurs two weeks later for *VvAdh2* (Fig. 2 A). Transcript levels during the preveraison growth phase were found to be very low. Some transcripts were detected in inflorescences and mature flowers. No, or very few, transcripts were detected in the vegetative tissues of embryogenic callus. *VvGrip4* transcript accumulated to similar levels in both Shiraz skin and flesh (Fig. 3 B).

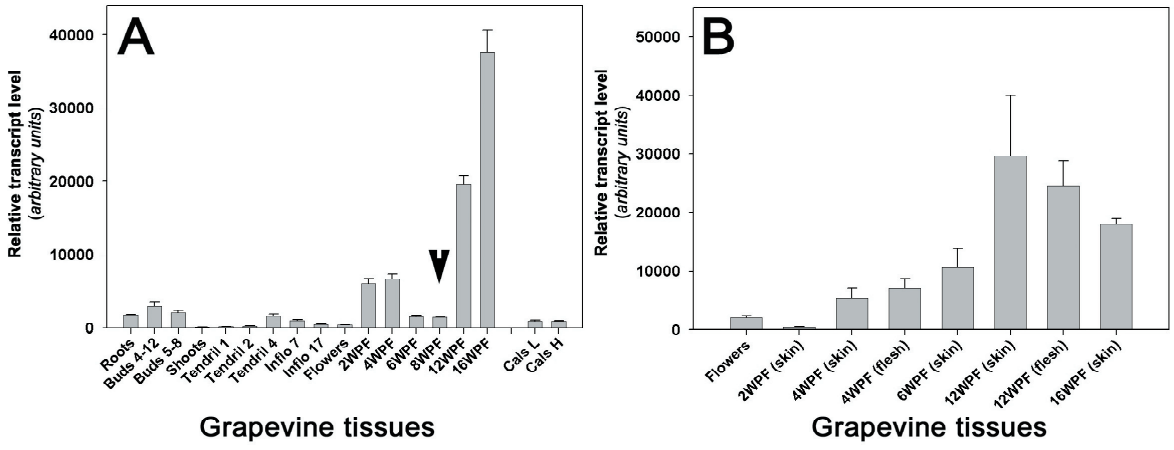

Fig. 2: Quantitative real-time PCR analysis of *VvAdh2* gene transcript accumulation for different stages of development (**A**) of various grapevine tissues and (**B**) in skin and flesh tissues of grape berries. For details see Fig. 1.

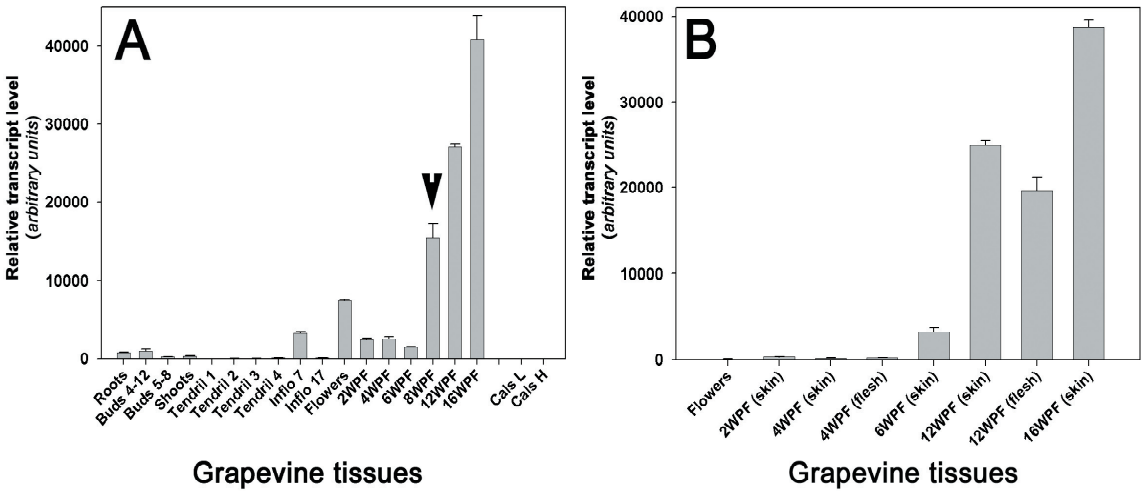

Fig. 3: Quantitative real-time PCR analysis of *VvGrip4* gene transcript accumulation for different stages of development (**A**) of various grapevine tissues and (**B**) in skin and flesh tissues of grape berries. Values for stages 12 week-post-flowering (WPF) and 16 WPF were divided by 100. For details see Fig. 1.

T a b l e

Primers used for real-time PCR experiments

Pair name	Primer F seq.	/stop codon	Primer R seq.	/stop codon	Product length
VvAdh1 F/R	tccgttctcagagatcaacaa	- 79	actctctcatctcaagatattctatgg	+ 21	103 bp
VvAdh2 F/R	attccagtcggcataagtgt	+ 20	ttgcaactgcatagacattgtt	+ 170	150 bp
VvGrip4 F/R	atgtataccgacgccacaa	+ 17	ctcatggactacaagcaaagaa	+ 167	150 bp
VvUbiq F/R	agtagatgactggattggaggt	- 2	gagtatcaaaacaaaagcatcg	+ 174	179 bp

Discussion

Transcripts of the three genes studied, *VvAdh1*, *VvAdh2* and *VvGrip4* accumulated to higher levels in berries than in the other tissues. Low levels of transcripts were found in all other tissues, except for tendril where no *VvGrip4* message was detected or in callus where no *VvAdh1* and *VvGrip4* transcripts were detected. *VvAdh1* transcripts accumulate predominantly during stage I of berry development and *VvAdh2* and *VvGrip4* transcripts are higher during ripening, confirming previous reports (Davies and Robinson 2000, Tesniere and Verries 2000, 2001). In addition, analysis of the same samples allowed different patterns of expression to be distinguished, *e.g. VvGrip4* transcript levels increased earlier in ripening than those of *VvAdh2*. However, the specificity of expression was not strictly defined as, *e.g.* transcripts for all three genes were detected at some level throughout berry development. This is in apparent conflict with earlier reports but may result from the higher sensitivity of real-time PCR analysis compared to northern hybridisation as used in previous studies.

The new data on gene transcript accumulation in skin *vs* flesh are very interesting since (1) these two tissues result from very different processes of development, and (2) they exhibit strong metabolic differences and have different functions. This study showed that these differences in tissue development and biochemistry did not result in a greatly differential pattern of transcript accumulation of *VvAdh1*, *VvAdh2* and *VvGrip4* genes.

VvGrip4 appears to be considerably more abundant than *VvAdh1* and *VvAdh2*. Its transcripts accumulate to very high levels during berry ripening as shown by differential screening (Davies and Robinson 2000) and by the frequency of isolation during EST sequencing. *VvGrip4* is similar to a hydroxyproline-rich, extensin-like protein from sunflower, but with a structure closer to that of nodulins (Davies and Robinson 2000). It is not clear why *VvGrip4* is up-regulated during fruit development. It possibly provides additional strength to the cell wall during rapid expansion of the cell and/or to restrict the invasion of pathogens. As the skin is more prone to pathogen contact than pulp one might have expected a higher level of *VvGrip4* expression in the external part of the berry but this is not the case.

This study presents new information regarding the expression of *VvAdh1*, *VvAdh2* and *VvGrip4* during the development of various non-berry organs including inflorescence and embryogenic callus. Even though the levels of transcript accumulation are highest in the berry, the patterns suggest that the three genes could also be involved in the development of other organs to a lesser extent.

The different patterns of transcript accumulation observed between the three genes suggest that different regu-

latory mechanisms are involved in the control of gene expression at the transcriptional level. Previous studies have shown differences in the promoter activity of *VvAdh1* and *VvAdh2* (Torregrosa *et al.* 2002, Verries *et al.* 2004). As the levels of transcripts of the three genes are very high in berries, the use of the corresponding promoter sequences could be useful to drive ectopic gene expression in this organ through genetic transformation. Depending on the characteristics of the target gene, the knowledge of the specificity and strength of any potential promoter sequence during the entire development of the grapevine plant, including the somatic embryo, is very important in the selection of the most suitable promoter sequence. Knowing the transcript levels in somatic embryo tissue is especially important as this is the preferred tissue for producing transgenic grapevine plants. Avoiding transgene expression in somatic embryos may be critical for some gene constructs that perturb normal cell function. It may also be useful to target the expression of genes to particular periods of development to prevent any unwanted consequences of gene overexpression and to increase the effectiveness of the transgene expression. Control of the level of transgene expression is also an issue of interest. More transcript is not always necessarily better and a number of circumstances can be envisaged where control over the level of transcript could be a significant advantage. Gene discovery in grapevine is progressing rapidly, so are the opportunities for using gene constructs with improved promoters.

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