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# The stimulation by ethylene of the UDP glucose-flavonoid 3-O-glucosyltransferase (UFGT) in grape tissues is independent from the MybA transcription factors

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1           **The stimulation by ethylene of the UDP glucose-flavonoid 3-O-**  
2           **glucosyltransferase (UFGT) in grape tissues is independent from the**  
3           **MybA transcription factors**

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10  
11           **Introduction:** Grapes are regarded as non-climacteric fruit (CHERVIN *et al.*, 2004 and  
12           references herein), in which ethylene evolution is very low and for which the ripening  
13           process seems to occur independently of ethylene production. However a recent study has  
14           shown that grape berry tissues have a fully functional pathway for ethylene synthesis, that  
15           this pathway is activated just before veraison when red berries start to accumulate  
16           anthocyanins, and that ethylene perception is critical for some berry changes associated with  
17           ripening, including anthocyanin accumulation (CHERVIN *et al.*, 2004). This last conclusion  
18           was supported by the fact that anthocyanin accumulation in the berry skins was inhibited  
19           when a specific inhibitor of ethylene receptors, 1-methylcyclopropene was applied to berries  
20           just before veraison, at the same time as ethylene production was activated in the berry.

21           The control of anthocyanin accumulation during the ripening phase in red grape  
22           berries is thought to be greatly dependent on UFGT activity (KOBAYASHI *et al.*, 2001;  
23           YAKUSHIJI *et al.*, 2006). This enzyme plays a key role in stabilising the aglycone moiety of  
24           the anthocyanins (PIFFAUT *et al.*, 1994) and may be essential for their transport to the  
25           vacuole. We have shown previously that exogenous ethylene could induce grape *ufgt*

1 expression (EL-KEREAMY *et al.*, 2003), confirming observations about the commercial use  
2 of ethylene precursor to boost grape skin colour.

3 The anthocyanin biosynthesis in model plants for the anthocyanin pathway such as  
4 petunia and maize is controlled by Myc and Myb transcription factors, and indeed, one  
5 transcription factor of this family, MybA1, was shown to be critical for the regulation of *ufgt*  
6 expression (YAKUSHIJI *et al.*, 2006 and refs therein) and anthocyanin accumulation in grape  
7 tissues. Walker *et al.* (2007) showed that two isoforms of this transcription factor, MybA1  
8 and MybA2 are involved in anthocyanin accumulation in grapevine.

9 The question remains whether the ethylene signal is acting on grape *ufgt* expression  
10 through the activation of *mybA* expression or via ethylene responsive transcription factors.

11

12 **Materials and methods:** Cabernet Sauvignon grapevines used for the ethylene  
13 experiments are grafted on 110 Richter rootstocks and grown in Toulouse, South-West of  
14 France, in a non-irrigated vineyard. Full bloom occurred around mid-June. The ethylene was  
15 applied for 24 h at different times after full bloom, at an initial concentration of 40  $\mu\text{L.l}^{-1}$ ,  
16 three biological replicates were then collected and stored at  $-80^{\circ}\text{C}$ . All other sampling  
17 processes and the RNA extractions were performed as described in EL-KEREAMY *et al.*  
18 (2003). Suspensions of purple grape cells, cv. Gamay were grown as described previously  
19 (TRANTAPHYLIDES *et al.*, 1993). Cells were vacuum-filtered onto Whatman n°1 filter paper  
20 and set upon 5 ml fresh MS-based cell culture medium in small Petri dishes. Cells were then  
21 sprayed with one ml of a sterile solution of 7 mM 2-chloroethylphosphonic acid (2-CEPA),  
22 a precursor of ethylene, the controls were sprayed with sterile water. Incubation time was 6  
23 hours before freezing samples for RNA extraction, performed as described above.

24 The qRT-PCR analyses were performed according to EL-SHARKAWY *et al.* (2005), with the  
25 following modifications. Oligonucleotide sequences of *mybA* primers were *VvmybA1c*(F), 5'-  
26 GCAAGCCTCAGGACAGAA -3' and *VvmybA1c*(R), 5'-

1 AAGCCCACATCAAATGGAAAA-3'. They would amplify both *VvmybA1* (AB097923)  
2 and *VvmybA2* (AB097924) isoforms. Oligonucleotide sequences for the *ufgt* primers were  
3 *Vvufgt*(F) 5'-GGCTTTTGTACACATTGCG-3' and *Vvufgt*(R) 5'-  
4 AAAAAGGGCCTGCAAATCAA-3'. The *myb* et *ufgt* mRNA levels were expressed as  
5 transcript accumulation indexes relative to a control gene *eflα*, as previously shown  
6 (TERRIER *et al.*, 2005). The LSD value between treatments was calculated at the 5% level  
7 using a one way ANOVA (SigmaStat, Systat Software Inc., San Jose, CA).

8 The ethylene cis-elements were estimated by homology search using PLACE  
9 database, <http://www.dna.affrc.go.jp/PLACE/signalscan.html> or PlantCARE database,  
10 <http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>.

11  
12 **Results and Discussion:** The application of gaseous ethylene significantly  
13 stimulated the accumulation of *ufgt* mRNA in berries when applied 8 and 9 weeks after full  
14 bloom (Fig. 1a). These observations made in Cabernet Sauvignon berry tissues were  
15 confirmed in another cultivar, under different experimental conditions, the Gamay cell  
16 suspension sprayed with the 2-CEPA, an ethylene precursor (Fig. 1c). These results  
17 confirmed previous observations, either the enhancement of anthocyanin accumulation after  
18 spraying 2-CEPA onto the grape clusters (EL-KEREMMY *et al.*, 2003), or the inhibition of  
19 anthocyanin accumulation following a treatment of the clusters with the ethylene inhibitor,  
20 1-methylcyclopropene (CHERVIN *et al.*, 2004).

21 However no significant difference was seen for the accumulation *mybA* mRNAs in  
22 the same experimental conditions (Fig. 1b and 1c). This absence of responsiveness to an  
23 ethylene signal was further confirmed by the analysis of the *VvmybA1* promoter, GenBank  
24 AB242302 (the sequence of the *VvmybA2* promoter was not available when we wrote this  
25 article), which showed three ethylene cis-elements (ethylene response elements, ERE) in the

1 -2 kb upstream region (Fig. 2a). The closest GCCGCC box, one of the most potent EREs  
2 (FUJIMOTO *et al.*, 2000), locates around -2100 bp. The closest ATTTnAAA box, the other  
3 common ERE, locates at -850 bp from *mybA1* start codon (Fig 2). In comparison there are  
4 seven EREs in the *ufgt* promoter, GenBank AY955269, (Fig. 2). The GCCGCC sequence  
5 was found at -160 bp from the *ufgt* start codon, a good place to be active (Dr Ohme-Takagi,  
6 pers. comm. and analyses of ethylene responsive promoters, data not shown). Two other  
7 ERE sequences, ATTTnAAA are reasonably close to the gene start codon, located between -  
8 450 and -350 bp. In another ethylene responsive promoter, they were shown to be active  
9 when placed at -500 bp (Itzhaki *et al.*, 1994). Here we used the sequence of the Shiraz *ufgt*  
10 promoter, which has 97 to 99 % homology to the grapevine *ufgt* promoter sequences isolated  
11 from the Kyoho, Italia, Ruby Oku, Muscat of Alexandria and Flame Muscat cultivars  
12 (KOBAYASHI *et al.*, 2001), and 95 % to Cabernet Sauvignon (GenBank AY919624). The  
13 alignment of various *ufgt* promoter sequences has already been showed (KOBAYASHI *et al.*,  
14 2001).

15

16 So we conclude that the stimulation of *ufgt* expression by ethylene is independent  
17 from *mybA* expression. But it does not preclude the importance of MybA in controlling the  
18 *ufgt* expression. Indeed, Dr Goto-Yamamoto found that the MybA protein binds to a region  
19 of the *ufgt* promoter around -428 to -303 bp from the start codon (pers. comm.) and recent  
20 works confirm the role of MybA in stimulating anthocyanin accumulation (Yakushiji *et al.*,  
21 2006; Walker *et al.*, 2007).

22 To our knowledge this is the first report addressing the responsiveness of *myb*  
23 expression to an ethylene signal.

24

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3
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13

#### 14 **Figure captions**

15 Figure 1: Effects of exogenous ethylene to a) the *ufgt* mRNA levels, to b) the *mybA1* mRNA  
16 levels in grape berries, cv. Cabernet Sauvignon according to time after full bloom, and to c)  
17 mRNAs of both genes in grape cell suspensions, cv. Gamay. The mRNA levels were  
18 determined by qRT- PCR analyses and are expressed as the transcript accumulation index  
19 relative to *efl- $\alpha$* . Each bar represents the mean of three biological replicates, and LSD bars  
20 were calculated at the 0.05 level for differences between the treatments (control or ethylene).

21

22 Figure 2: Locations of ethylene responsive elements (cis-acting) on the promoter of *mybA1*  
23 (AB242302) and on the promoter of *ufgt* (AY955269) of *Vitis vinifera*. The nucleotide  
24 numbering is relative to the start codon (0). Cis-elements were estimated by homology  
25 search using PLACE and PlantCARE databases.

1

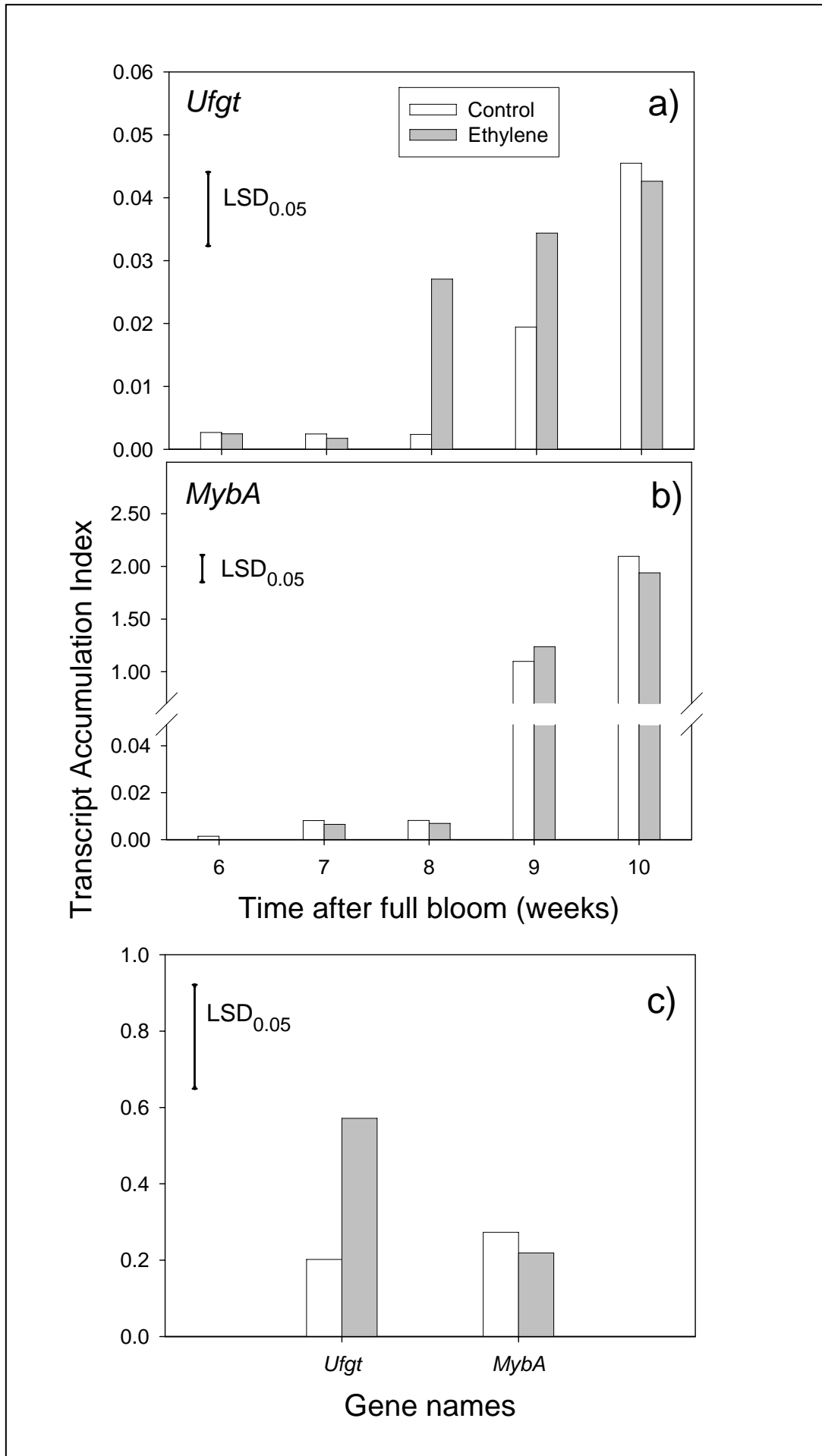


Figure 1



1

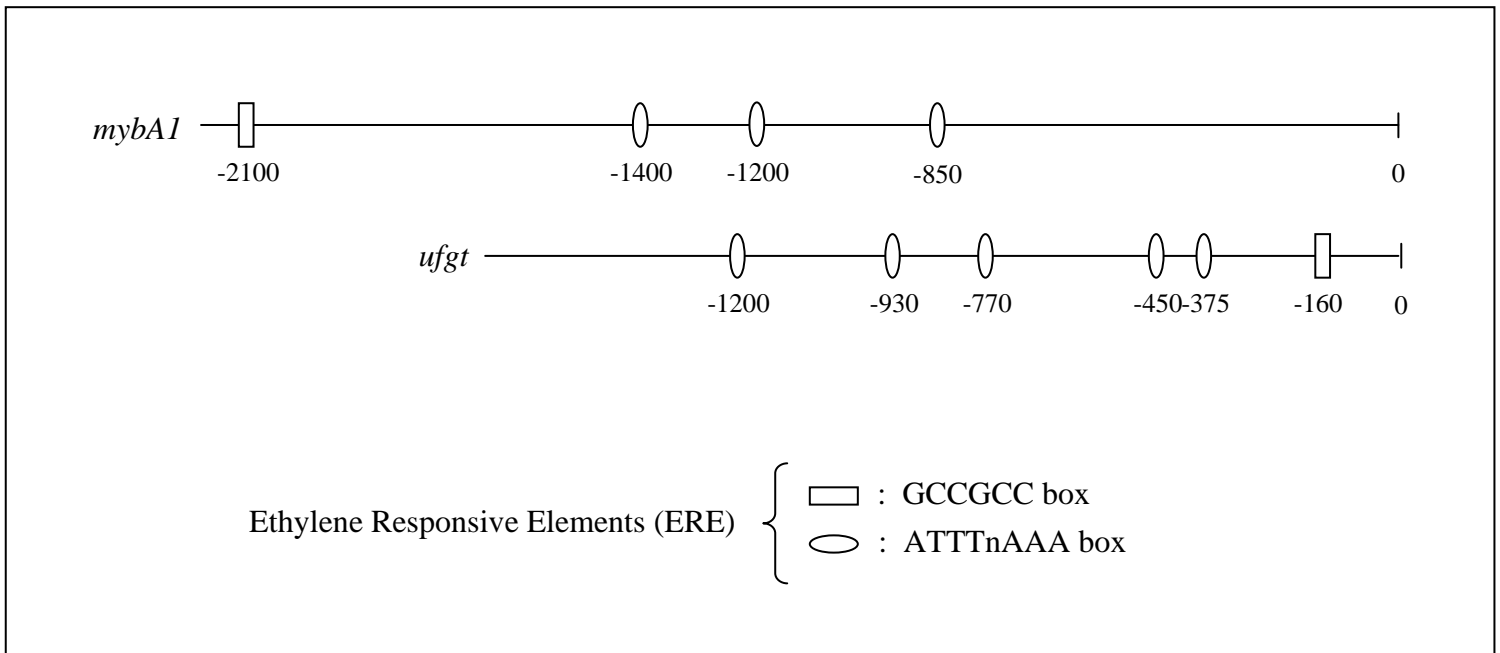


Figure 2