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Ethylene and other stimuli affect expression of the UDP glucose-flavonoid 3-O-glucosyltransferase in non-climacteric grape cells

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Summary

The UDP glucose-flavonoid 3-O-glucosyltransferase (UFGT) is a key enzyme for anthocyanin biosynthesis and stability, the pigments of red grapes. The understanding of factors affecting expression of this enzyme is important for the control of grape colour. A 1640 bp promoter region of the *ufgt* gene was cloned and sequenced. The analysis of this sequence revealed seven putative ethylene-responsive cis-elements and other cis-elements related to three major signals known to induce anthocyanin accumulation in plant tissues: light, sugar, and abscisic acid. In order to evaluate the ability of ethylene and other signals to drive expression from the *ufgt* promoter, we ran transient expression experiments using an anthocyanin-rich grape cell culture, with very low green auto-fluorescence. After biolistic bombardment, the cells were treated to test various combinations of the four signals on *gfp* expression (green fluorescent protein).

The comparison of the intensity of fluorescent light accumulated in cells following the various treatments showed that ethylene better stimulates expression of the *ufgt* promoter in the dark than with light. In addition, results showed that there may be a positive interaction between ethylene and abscisic acid. This system, a promoter of interest driving the *gfp* expression in cells with low auto-fluorescence, may be a good tool for studies about synergistic or antagonist roles of transcription factors. Moreover, a specific inhibitor of ethylene receptors (1-methylcyclopropene) inhibited *ufgt* mRNA accumulation in grape berries. This confirms that the ethylene signal is likely a regulator of grape UFGT expression in a non-climacteric fruit.

Keywords: UDP glucose-flavonoid 3-*O*-glucosyltransferase (UFGT), grape, anthocyanins, ethylene, ethylene receptors.

Introduction

Ripening is an essential step of fruit development that conditions the qualities of both whole fruit and transformed products. There are two classes of fruit with regards to their ripening behaviour: the climacteric fruit for which ripening steps are controlled by a relatively strong ethylene evolution, and the non-climacteric fruit in which ethylene evolution is very low and for which the ripening process seems to occur independent of ethylene production (GIOVANNONI 2001). Grapes are regarded as non-climacteric fruit (COOMBE AND HALE 1973). However a recent study has shown that grape berry tissues have a fully functional pathway for ethylene synthesis, and that this pathway is activated just before véraison (CHERVIN *et al.* 2004), when berries start to soften, accumulate sugars and, in red cultivars, accumulate anthocyanins. Furthermore this study showed that ethylene perception is critical for some berry changes associated with ripening, including anthocyanin accumulation (CHERVIN *et al.* 2004). This conclusion was supported by the fact that anthocyanin biosynthesis in the berry skins was inhibited when a specific inhibitor of ethylene receptors, 1-methylcyclopropene or 1-MCP (BLANKENSHIP AND DOLE 2003), was applied to berries just before veraison, at the same time as ethylene was produced in the berry (CHERVIN *et al.* 2004).

The control of anthocyanin accumulation during the ripening phase in red grape berries is thought to be greatly dependent on UFGT activity (BOSS *et al.* 1996, KOBAYASHI *et al.* 2002, KOBAYASHI *et al.* 2004). This enzyme plays a key role in stabilising the aglycone moiety of the anthocyanins (PIFFAUT *et al.* 1994) and may be essential for their transport to the vacuole. To investigate which factors induce grape *ufgt* expression, we cloned and sequenced the grape *ufgt* promoter. We found different cis-element motifs including ethylene responsive elements (ERE). The *ufgt* promoter was fused to the *gfp* reporter gene and various transient expression studies in grape cell suspensions were conducted in order to test the importance of ethylene signals and other stimuli in the control of transcription from this promoter. We completed previous results indicating that exogenous ethylene could induce grape *ufgt* expression (EL-KEREAMY *et al.* 2003), and confirming observations about the use of ethylene precursor to boost grape skin colour (WEAVER AND MONTGOMERY 1974). We then evaluated in grape berries whether the ethylene signal was acting on grape *ufgt* expression directly via ethylene receptors or indirectly through alternate pathways. These results confirm the role of ethylene signals in the control of the *ufgt* expression in a non-climacteric fruit and present a method that could be a good tool to study steric hindrance of transcription factors on a promoter.

Materials and Methods

A subclone obtained from the 5'-end of a grapevine *ufgt* cDNA (FORD *et al.* 1998) was used to probe a 400 000 plaque aliquot of a genomic library made from the cultivar Shiraz. Two positive plaques were obtained and one was used to isolate a genomic fragment consisting of 736 bp of *ufgt* coding sequence and 1647 bp of promoter sequence immediately upstream of the ATG start codon. For expression analysis, the grapevine *ufgt* promoter region was amplified using the primers UTPPF (5'-TCCCCGGGCTTTTCGGTATCATGCGTCC-3') and UTPP2 (5'-TCCCCGGGTTGGAATGGGGGATGTT-3') and cloned into a promoterless-gfp shuttle vector (pART7napx+GFP) developed from pART7 (GLEAVE 1992) and pBINm-gfp5-ER (HASELOFF *et al.* 1997). The entire expression cassette was then cloned into the binary vector pART27 (GLEAVE 1992) and the final construct was called pVvufgt::gfp. The cis-elements were estimated by homology search using PLACE database, <http://www.dna.affrc.go.jp/PLACE/signalscan.html> (HIGO *et al.* 1998) or PlantCARE database, <http://intra.psb.ugent.be:8080/PlantCARE/> (LESCOT *et al.* 2002). Comparisons were made between the results obtained before and after the randomisation of the promoter sequence using the same databases. When the number of repetitions of one cis-element was equal or higher in the randomised sequence than in the original one, we kept it in the figure, but showed its name in italics.

Suspensions of purple grape cells, cv. Gamay, initially obtained from grape berries, were grown as described previously (TRANTAPHYLIDÈS *et al.* 1993). Biolistic experiments were performed using a helium-driven gun with an initial propelling He pressure of 3.1 bars and a 30-mbar vacuum in the chamber at shooting time. The previously described protocol (TORREGROSA *et al.* 2002) was used with slight modifications. Briefly, cells were vacuum-filtered onto Whatman n°1 filter paper and set upon MS-based cell culture medium containing 60 mM sucrose and solidified with 3 g.l⁻¹ Phytigel in small Petri dishes. Gold microprojectiles (1.0 µm, Biorad) were coated with the pVvufgt::gfp plasmid to achieve 1 µg DNA delivery per shot. Experiments were performed twice (two different dates), each date with three Petri dishes of 55 mm diameter (one dish per shot) for each treatment or treatment combination in each instance. After shooting, cells were sprayed with sterile water (control) or one ml sterile solutions of either 7 mM 2-chloroethylphosphonic acid (2-CEPA), a generator of ethylene, or 150 mM sucrose or 500 µM abscisic acid (ABA), each compound added alone or in all combinations. To test the influence of darkness, the Petri dishes were wrapped in aluminium foil. The incubation time to get optimal

GFP signal was around 40 h in a growth chamber with a 16:8 Light:Dark illumination cycle. The observations were made in the morning of the second day after shooting, 2 h after light resumption. Image acquisition and analysis were performed according to CORMEAU *et al.* (2002), with the following modifications. The samples were examined with an epifluorescence microscope (DMIRB-E, Leica, Germany) equipped with suitable excitation and emission filters (i.e., blue range excitation BP 450-490 nm, DM 510 nm, LP 515 nm). Images were acquired using a Color CoolView 3-chip on chip CCD camera (Photonic Science, Millham, UK). The camera settings were identical for all experiments. For each image, the fluorescence intensity within the cell and the background were both measured from the green channel, using Image Pro-Plus 4.5 (Media Cybernetics, Silver Spring, MD, USA). The fluorescing cells were spotted by examining the Petri dishes with 10X magnification. The images of all observable cells per Petri dish were captured. A total of six Petri dishes were observed per treatment (three replicates x two bombardment series). There was no indication of the number of plasmids inserted per cell, but this is likely to vary similarly between treatments, as these were performed after the bombardment. The quantification was performed using SigmaScan (SPSS Inc., Chicago, IL). The signal intensities were normalised so the mean of controls under light was equal to 100. The LSD value was calculated at the 5% level using a one way ANOVA (SigmaStat, SPSS, Chicago, IL).

The grape berries used for the 1-MCP experiments were from Cabernet Sauvignon grapevines grafted on 110 Richter rootstocks and grown in Toulouse, South-West of France, in a non-irrigated vineyard. Full bloom occurred around mid-June. The 1-MCP was applied using a polyethylene bag wrapped around the cluster, for 24 h, once a week, at various times following full bloom. The initial 1-MCP concentration was $4 \mu\text{l.l}^{-1}$. Control clusters were wrapped in plastic bags for 24 h. For these experiments, clusters growing in a shaded area of the vines were chosen to avoid direct exposure to sunlight and overheating associated with such a treatment. After treatments, the clusters were sampled and stored at -80°C pending further analyses. Northern and Western blots were performed as previously described (EL-KEREAMY *et al.* 2003).

Results and Discussion

In order to investigate the effects of ethylene and other possible stimuli on *ufgt* expression, a genomic clone encoding the promoter of grapevine *ufgt* was isolated and a 1647 bp region upstream of the putative ATG translational start codon cloned. The Shiraz *ufgt* promoter sequence isolated in this study (GenBank AY955269), has 97 to

99 % homology to the grapevine *ufgt* promoter sequences isolated from the Kyoho, Italia, Ruby Oku, Muscat of Alexandria and Flame Muscat cultivars (KOBAYASHI *et al.* 2001), and 95 % to Cabernet Sauvignon (GenBank AY919624). The alignment of various *ufgt* promoter sequences has already been showed (KOBAYASHI *et al.* 2001). On all these promoter sequences, one ethylene cis-element, initially reported (ITZHAKI *et al.* 1994), attracted our attention. This ERE sequence, ATTTCAA is located at -365 bp (Fig. 1). This element was also present with a sequence differing by a single nucleotide, ATTTTAAA, at three other sites that could also be potential EREs. Indeed, this latter sequence is recognised as a potential ethylene cis-element using the online-database, PlantCARE (LESCOT *et al.* 2002). Furthermore we found a promoter region with sequence homology to another class of ethylene cis-elements at -160 bp (sequence GCCGCC) previously described by FUJIMOTO *et al.* (2000). The presence of several putative ethylene cis-elements in the grapevine *ufgt* promoters is evidence to support our observations about the potential role for ethylene in inducing *ufgt* expression in grape berries after veraison. The EREs mentioned above are also present in the Cabernet Sauvignon *ufgt* promoter. Submitting this promoter to PLACE analysis (HIGO *et al.* 1998), we found many sequence matches to other known cis-elements (Fig. 1), with several corresponding to three other stimuli known to affect plant development or metabolism: light, sugar and abscisic acid. These are also signals known to induce anthocyanin accumulation in grapes and other plant tissues (MOL *et al.* 1996).

Using the Shiraz *ufgt* promoter transcriptionally fused to the *gfp* gene, we carried out transient expression experiments using purple grape cell suspension cultures established from the berries of the grape cultivar Gamay. These cells have been shown to produce glycosylated anthocyanins (AFIFI *et al.* 2003), thus we expected that all the components necessary to activate the glycosylation of anthocyanins via the *ufgt* gene were present in these cells. These purple cells also present the advantage of having very low auto-fluorescence at the wavelength used to track GFP activity. The aim of this transient expression study was to check the importance of ethylene signalling in inducing *ufgt* expression along with the three other potential regulators of *ufgt* expression mentioned above. We tested the effects of darkness or light alone on reporter gene expression, the addition of single effectors (ABA, ethylene or sucrose) on GFP fluorescence, and then the influence of all double and triple combinations of the stimuli on GFP expression. Figure 2 shows that ethylene stimulates GFP production in the transient assays in darkness (a significant increase of the GFP signal by 25 % compared

to the dark control alone), but ethylene does not stimulate GFP expression from the *ufgt* promoter in light (a non-significant increase by 5 %). This absence of stimulation in light could be due to overlapping or proximal cis-elements (one LRE and one ERE) like those at -366 bp or - 420 bp or -766 bp or - 936 bp (Fig. 1). This is the case of nearly 60 % of the EREs located on this promoter. This type of inhibition may be due to steric hindrance of transcription factors as discussed by (HAHN *et al.* 2003). A region from -428 to -303 bp seems to anchor the MybA protein (N. Goto-Yamamoto, personal communication). The corresponding element could be a LRE as *mybA1* expression is light dependent (YAKUSHIJI *et al.* 2006 and refs therein). It is also possible that the timing of the initiation of each stimulus, leading to presence of transcription factors in a set order, may also lead to either synergistic or antagonist effects regarding transcription. In the dark, there is no additive effect due to ethylene in combination to other effectors (Fig. 2). The signal intensity reached a maximum as soon as one effector was present. In the light, ethylene and ABA may present some additive effect, as the signal intensity was significantly increased by + 11% in comparison to control (= light alone), when none of the single effectors (ethylene or ABA) led to an increased signal. As in other experiments of transient expression published in various plant systems, one can question their biological significance. In answer to this question, we can propose this set of results as a first description of the ethylene role in a complex combination of stimuli, controlling the expression of one key protein (UFGT) involved in the production of the anthocyanins. ABA also had a stimulatory effect on GFP expression in the transient assays carried out in the dark, but no significant change was seen when the cells were maintained in the light (Fig. 2). Such an ABA effect on *ufgt* expression in grape berries has been reported recently (Peppi *et al.*, 2008). Sucrose treatments increased the levels of GFP fluorescence in both dark and light grown cells (Fig. 2). In the context of berry ripening, these results are most interesting. *ufgt* expression is induced at veraison and continues throughout the rest of berry ripening (BOSS *et al.* 1996). The induction of UFGT coincides with the initiation of an influx of sugars into berries and a peak of ABA levels in the berries (BOSS *et al.* 1996, DAVIES *et al.* 1997). Thus, it is possible that either of these stimuli may promote expression from the *ufgt* promoter in vivo. Light was also found to stimulate GFP expression from the Shiraz *ufgt* promoter in the transient assays (Fig. 2). This matches results obtained with Cabernet Sauvignon, in which shading of bunches has been shown to reduce the accumulation of anthocyanins (JEONG *et al.*, 2004).

The application of 1-MCP significantly inhibited the accumulation of *ufgt* mRNA in Cabernet Sauvignon berries when applied 10 weeks after full bloom (Fig. 3a). Western blot analysis was run for the times at which *ufgt* mRNAs were detected. No significant difference was seen for UFGT protein levels between control and 1-MCP treatments at each timepoint analysed; however 1-MCP treatment attenuated UFGT protein accumulation levels over the three sampling times (Fig. 3b). The fact that the 1-MCP effect was significant only on mRNA and not on proteins may be due to the higher stability of UFGT protein compared to mRNAs. To see changes in protein levels we may need to sample berries later than 24h after treatment to allow for the reduction in steady state *ufgt* mRNA levels to be reflected in reduced UFGT protein. Nevertheless, the difference seen in *ufgt* mRNA levels upon 1-MCP application in the 10 weeks post flowering sample confirms the previous observation that the induction of UFGT by exogenous ethylene is probably due to ethylene signal transduction and not to indirect stimulation via other metabolisms activated by ethylene or side-effects of the relatively high dose of the ethylene precursor that was sprayed onto the grapes (EL-KEREAMY *et al.* 2003). The inhibition of the UFGT protein accumulation by 1-MCP (Fig. 3b), without being significant by Western analysis, may lead to the significant decrease of anthocyanin accumulation previously observed (CHERVIN *et al.* 2004), as for week 10 there was a drop by almost 50 % of the protein accumulation after treatment with 1-MCP (Fig. 3b). The application of exogenous ethylene led to significant increases of both *ufgt* mRNAs and proteins (EL-KEREAMY *et al.* 2003), and this is consistent with the decrease of both messengers and proteins by an ethylene inhibitor (1-MCP) presented in this paper. The control of the anthocyanin biosynthesis pathway in plants has been shown to occur at the level of gene transcription (MARTIN AND GERATS 1993). Studies into the promoters of anthocyanin biosynthesis genes in both *Antirrhinum* (SABLOWSKI *et al.* 1994) and maize (ROTH *et al.* 1991) have shown that transcriptional controlling elements are usually present upstream of the transcription start site. In grapevine, the *ufgt* expression correlates with the anthocyanin biosynthesis, which suggests that important promoter elements upstream of this gene control anthocyanin production in this plant species (BOSS *et al.* 1996). The results of the 1-MCP treatments above and experiments from previous work (EL-KEREAMY *et al.* 2003) suggest that the *ufgt* promoter can respond to ethylene signalling.

In conclusion, the 1647 bp upstream region of the Shiraz grapevine *ufgt* promoter has been shown to contain cis-elements that respond to the correct stimuli required for UFGT and thus anthocyanin production in berry skins. Transient

expression analyses show that light, ABA, sugar and ethylene can all stimulate expression from the *ufgt* promoter under certain conditions (Fig. 1). This is consistent with known changes in berry composition during berry development and in experiments designed to alter these variables (BOSS *et al.* 1996, DAVIES *et al.* 1997, DOWNEY *et al.*, 2004, JEONG *et al.*, 2004). The trend observed here, that ethylene better boosts the *ufgt* expression in the dark than in the light, matches well the trend observed in the vineyards that spraying ethylene precursors has a more perceptible effect on clusters growing in shaded areas or during years with a low total sunlight hours over the ripening period (unpublished results). From this work and previous studies (CHERVIN *et al.* 2004, EL-KEREAMY *et al.* 2003, WEAVER AND MONTGOMERY 1974), it is quite clear that ethylene is involved in the signal mix leading to the *ufgt* expression in grape cells, and particularly in berry skin tissues that accumulate high concentrations of anthocyanins.

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Figure captions

Fig. 1: Sequence of the *ufgt* promoter from grapevine cv. Shiraz. The numbering odd nucleotide is relative to the start codon. Cis-elements estimated by homology search using PLACE database (HIGO *et al.* 1998) and PlantCARE (LESCOT *et al.* 2002) are indicated in bold letters (ABRE: ABA responsive element; ERE: ethylene responsive element; LRE: light responsive element; SURE: sugar responsive element). Cis-elements in italics were found with the same abundance or greater in a randomized sequence.

Fig. 2: Activity of the *ufgt* promoter as a function of various stimuli. Fluorescence of the GFP, driven by an *ufgt* promoter, after bombardment of grape cells cv. Gamay and spraying with water (control), or solutions of abscisic acid, or chloroethylphosphonic acid, an ethylene generator, or sucrose, and combinations of these stimuli. The cells were incubated in dark or light conditions. The bars represent the mean values of x individual fluorescing cells, x is given at the bottom of each bars. The numbers correspond to the means of six cell batches (3 replicates \times 2 dates). The normalization was performed by giving the “100” value to the mean value of “light controls”. The error bars represent SE, and the LSD bar was calculated at the 5% level.

Fig. 3: Effects of 1-MCP (methylcyclopropene), inhibitor of ethylene receptors, on the expression of the *ufgt* gene in grape berries, cv. Cabernet Sauvignon, as a function of the time of application after full bloom. A: Northern blots, results normalised with the 18S signals, the picture shows one of the three blots; B: Western blots, results normalised with the red Ponceau signals, the picture represents one of the three blots; $n = 3$ biological replicates, error bars represent SE, p is the probability that there was no difference between the control and the MCP means at the same sampling time (t-test).

ABRE SURE
 -1647 ATATTGACATGTGTAAAAAAAAT**AATAAAAA**TCAAATCTCATCTTGACATCCAATGATT LRE
 -1585 GGTATGAATTCAACAGTACCTAATAGCACAACTACTATAGAAATCTCCCTAAATTTAT**GAAAA**
 -1523 **AATTACATTTT**GTAAATCTATCTAACCTCTGATTCAGCACACTCTAGAGCAACATTGAGA
 ABRE ERE
 -1461 **CAACTGCAGGCTGCAGCTTTT**TCAACAATCTCAATTTT**GCCCCAAAAATTTCCAAA**CATGGA
 -1399 **AACCAGAAAA**TATTGTGACTTGTAAAGGGCTTTTTCGTCATTT**CA**TCTTGTTTTATTTGAC LRE
 -1337 ATCAATTTTTTTAAATTGCTTTTAAGAACAAAAATTTATCTAGGAACATAAAATAT**GAAAAACG**
 ABRE SURE
 -1275 ATATTTTGGTCTTAT**CCACATG**AGGGTGTTGTATAATTATAT**GCAATAAAAAAA**TTGTTGA
 LRE ERE ABRE
 -1213 **AATATTTT**TCAT**ATTCAATTG**TTATCTAAAAACTTTACAAAAAAACGATTGAGAGCATAT
 -1151 TAGACTTGGTCTTAAAAATAACTTGTTTTTAGAACACATTCTCAAAAAAATAATTTTCTGTA
 ABRE
 -1089 **AAAAATTT**AGGGTCTGTTTATAACTATTTTCTAAAA**CAGTTA**TAAAAAATAGTTTTTTAAA
 -1027 **ACTTTTCT**CTAATGTTTTGTAAACAAAAATTTGTTTAATAATCTAAAAATTTTAAACATAT
 LRE ERE LRE
 -965 TTTAAATATTTTTTTTAGTAT**TATTTTAAA**ATAAGTTTTATCTAGTATTT**TATTTT**TAAT
 LRE ABRE ABRE
 -903 CATTCTACATATTTGTATAAT**TATTTT**TCAAATA**TAACCA**TAAAAAAAC**AGTTAA**AAA GAAATT
 LRE
 -841 TTCT**GAAAAT**ACCGTATTTTTTTTATCTAAAAGTTTTTTATTGTAAATATATTATCCTAG
 LRE ERE SURE ABRE LRE SURE
 -779 TTTTT**TATTTTAA**GAATAGAAA**ACCA**TTCTCG**GAAAA**TAAATACCAAATAGACTCTTACTT
 -717 TTTAAATAAAAATACAGTTTTTGGGTCTTTTCAAAAAAGTTTTCAAAACAAGTCCTTTAGTT LRE
 -655 TCTTTAATTATTTGCTTTTTCAAAATCAATCTTTAAAAATATTTTT**GAAAATAA**TTCTTT
 ABRE ABRE
 -593 AAAATATTTTT**CAATTG**TTTTTGGTAACAAAAATCTATCTAA**TAACCA**AAAATAAAAAAT
 ABRE
 -531 ATTTTTTTAGTTCTCTTCAAAAAGTAATATATAT**CAATGTGG**AAACAAAACTTTAAAA
 ERE LRE
 -469 CATTCTACCTTTTCAATATTACTAAAAAACA**ATTTTAA**TTATTCTTAAAA**CAATGGG**
 LRE ERE
 -407 GTTTTAATCAAATTAATTTTGAACAATTAATTT**TATTTCA**AAAAATTTATTGAATCATATTT
 SURE LRE LRE
 -345 TTCAAATTAGAAAA**CA**ATTTTATGTTGTTTGA**ATAGAAA**TTATTTT**G**AAAGTAAAATTA
 LRE CCAATbox
 -283 CAAATATGCTCTTGCTACTACTCGCC**CAAGTATATCCCA**CC**CAATGG**CAAAAGTAAAAGCTCACA
 ABRE ERE
 -221 CAGAGCTTTCACTTGCCCGTGGTTTTGTTTT**TTTTTCCCA**TTTTTTCTTTCTTTGGCCGC
 ABRE LRE
 -159 **ATGCAGAA**TGGTGGTTTGGTTTTGGTTGGTTTGTAGGAGGGTGGGAATTGGGATGACAAC
 ABRE ABRE ABRE ABRE TATbox
 -97 CCCC**CATGCAGTTG**CCACTCTCACAACCCCC**CATGCAGTTG**CTCTCAATTTATAATCTTCAACAG
 -35 CCAAAACCAAATTGTAACATCCCCCATCCAACCATG

Fig. 1

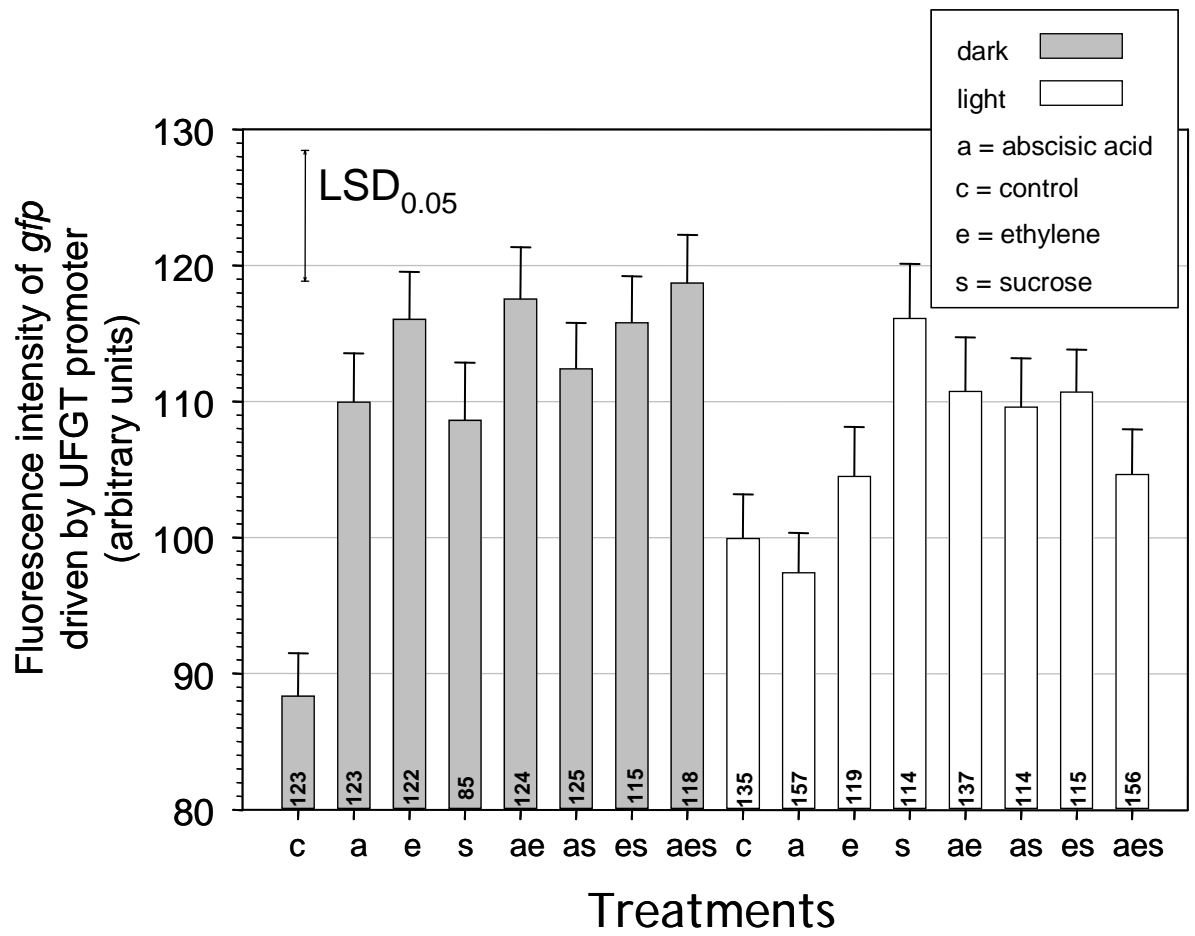


Fig. 2

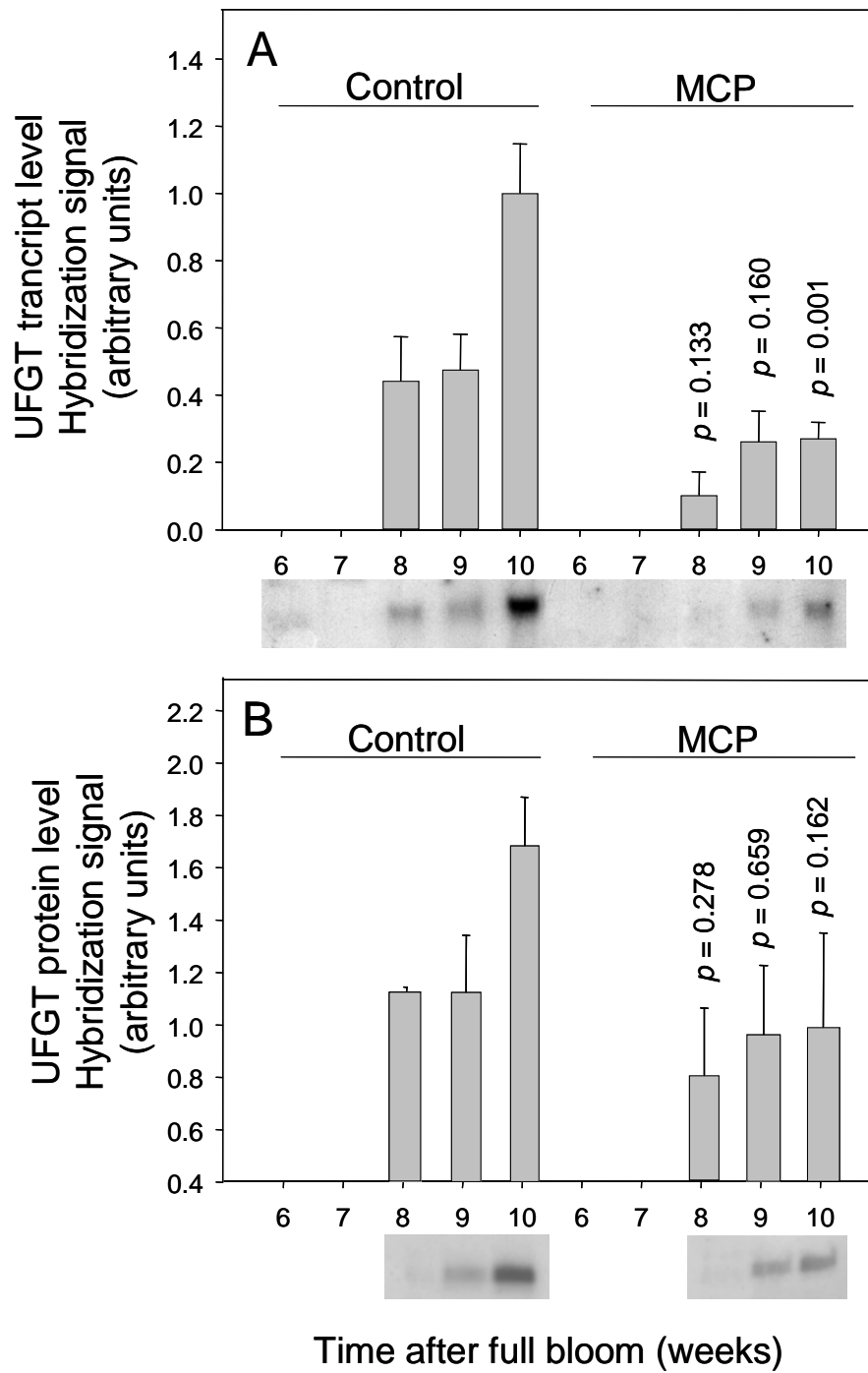


Fig. 3