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

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Summary

The UDP glucose-flavonoid 3-O-glucosyltransferase (UFGT) is a key enzyme for biosynthesis and stability of anthocyanin pigments of red grapes. Understanding factors affecting expression of this enzyme is thus important for the control of grape colour. A 1640 bp promoter region of the grapevine ufgt gene was cloned and sequenced. Sequence analysis revealed seven putative ethylene-responsive cis-elements and others 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 with various combinations of the four signals on gfp expression (green fluorescent protein). The comparison of fluorescent light intensity in cells subjected to the various treatments showed that ethylene better stimulates expression of the ufgt promoter in the dark than under light. In addition, results showed that there may be a positive interaction between ethylene and abscisic acid. This system, a promoter of gfp expression in cells with low auto-fluorescence, may be a good tool for studies about synergistic or antagonist roles of transcription factors. Moreover, treatment of grape berries with a specific inhibitor of ethylene receptors (1-methylcyclopropene) inhibited ufgt mRNA accumulation. This confirms that the ethylene signal is likely a regulator of grape UFGT expression in a non-climacteric fruit.

Key words: anthocyanins, cis-elements, ethylene, ethylene receptors, grape, promoter, UDP glucose-flavonoid 3-O-glucosyltransferase (UFGT).

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 fruits with regard to their ripening behaviour: climacteric fruits for which ripening steps are controlled by a relatively strong ethylene evolution, and non-climacteric fruits in which ethylene evolution is very low and for which the ripening process seems to occur independently of ethylene production (GIOVANNONI 2001). Grapes are regarded as non-climacteric fruits (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 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 véraison, at the same time as ethylene was being 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 anthocyanins (PIFAULT 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, which revealed different cis-element motifs including ethylene responsive elements (ERE). The ufgt promoter was then 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 thus complemented 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 enhance grape skin colour (WEAVER and MONTGOMERY 1974). We then evaluated in planta whether the ethylene signal was acting in grape berries on 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.
Material 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'-TCCCCGGGTTCG-GATCATCGCTCC-3') and UTPP2 (5'-TCCCCCGGTT-GGAATGGGGATGTT-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 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 (indicating a highly probabilistic unspecificity), we kept it in the figure, but showed its name in italics.

Suspensions of purple grape berry cells, 'Gamay' were grown as described previously (Triantaphyllides 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 sterile Whatman n°1 filter paper and set upon MS-based cell culture medium containing 60 mM sucrose and solidified with 3 g·l⁻¹ Phytagel. 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). Camera settings were identical for all experiments. For each image, the fluorescence intensity within the cell and in the background was 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 a 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 µl⁻¹. Control clusters were wrapped in plastic bags for 24 h. For these experiments, only clusters growing in a shaded area of the vines were chosen to avoid direct exposure to sunlight and overheating associated with such a treatment. A total of 5 clusters (each from a different plant) per treatment were used, with ten berries (2 from each cluster) pooled for analysis (three biological repetitions). After treatments, the clusters were 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), displayed 97 to 99 % homology to the grapevine ufgt promoter sequences isolated from 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 grape ufgt promoter sequences has already been shown (Kobayashi et al. 2001). From all these promoter sequences, one ethylene cis-element, initially reported by Itzhaki et al. (1994), attracted our attention. This ERE sequence, ATTTCAAA 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 thus also be potential EREs. Indeed, this latter sequence is recognised as a potential eth-
Ethylene and other stimuli affect expression of the UDP glucose-flavonoid 3-O-glucosyltransferase

Ethylene and other stimuli affect expression of the UDP glucose-flavonoid 3-O-glucosyltransferase 13 cis-element using the online-database, PlantCARE (Lescot et al. 2002). Furthermore we found a promoter re-... 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 véraison. 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 (light, sugar and abscisic acid) known to affect plant development or metabolism and in particular to induce anthocyanin accumulation in grapes and other plant tissues (Mo 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 berries of the Gamay grape cultivar. As these cells have been shown to produce glycosylated anthocyanins (Afifi et al. 2003), we thus 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. Fig. 2 shows that ethylene stimulated GFP production in the transient assays in darkness (a significant increase of the GFP signal by 25 % compared to the dark control alone),

Fig. 1: Sequence of the ufgt promoter from grapevine '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.
but ethylene did 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) such as 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 steri- hindrance of transcription factors as discussed by HAHN et al. (2003). 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 grape berries (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 véraison 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 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. 3 a). Western blot analysis was run whenever ufgt mRNAs were detected. No significant difference was seen in UFGT protein levels between control and 1-MCP treatments at each
time point analysed; however 1-MCP treatment attenuated UFGT protein accumulation levels over the three sampling times (Fig. 3 b). 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 be able to observe changes in protein levels we may need to sample berries later than 24 h after treatment to allow for the reduction in ufgt mRNA levels to be reflected in reduced UFGT protein level. 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. 3 b), 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 % in the protein accumulation after treatment with 1-MCP (Fig. 3 b). 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 provoked in both messengers and proteins content by an ethylene inhibitor (1-MCP) as 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 GERAT 1993). Studies into the promoters of anthocyanin biosynthesis genes in both Antirrhinum (SABLORSKI 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 anthocyanin biosynthesis, which suggests that important promoter elements upstream of this gene control anthocyanin production in this 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 indeed 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. 2). 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 enhances 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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