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Ethylene is Required for the Ripening of Grape

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Abstract

While grapes have been classified as a non-climacteric fruit, we show here that endogenous ethylene production just before veraison is required for an increase in berry size and possibly for anthocyanin accumulation in the ripening berry. Our data also show that the peak of ethylene production just prior to veraison is associated with increased accumulation of ACC oxidase mRNAs, enhanced ACC oxidase activity and higher concentrations of malonyl-ACC. Exposure of clusters to 1-MCP at various times before and after veraison inhibited ripening only in fruit treated at the time of the ethylene peak. Lastly, we observed some feed-back at the ethylene perception level and this response is discussed in relationship to the behaviour of non-climacteric plant tissues.

INTRODUCTION

Ethylene was thought to have had a very limited role, if any, in the ripening process of non-climacteric fruit (Coombe and Hale, 1973; Abeles et al., 1992). More recent work has indicated that some aspects of non-climacteric ripening may be associated with ethylene responses (Giovannoni, 2001). The classification of grapes as non-climacteric fruit was mainly due to a set of data showing only weak changes in endogenous ethylene levels around veraison (Coombe and Hale, 1973), a development stage often considered the beginning of ripening in grape berries (sugar accumulation increases, acid decreases, the berry softens and pigmentation occurs). Indeed, Coombe and Hale (1973) and Alleweldt and Koch (1977) found that the amounts of endogenous ethylene produced by grapes were quite small when expressed as a concentration per volume of internal gas (less than $0.5 \mu\text{l L}^{-1}$), but when expressed as a concentration per weight of tissue, then an ethylene burst was clearly observable around veraison (Alleweldt and Koch, 1977). However, the peak in the latter study represented one point (one date on which ethylene production rose) and the fruit was incubated for one hour under partial vacuum. This was an excessive period of time over which some of the ethylene collected could have been due to a plant response to the vacuum.

MATERIALS AND METHODS

'Cabernet Sauvignon' grapevines grafted onto '110 Richter' were grown in Toulouse, France, in a non-irrigated vineyard. Measurements were made over two consecutive years with anthesis occurring in mid-June. The 1-MCP at an initial concentration of $4 \mu\text{l L}^{-1}$ was applied at various times following anthesis, for a 24 h period, in a polyethylene bag wrapped around the cluster. Control clusters were wrapped with plastic bags for 24 h. Clusters located in a shaded area of the vines were chosen to

avoid direct exposure to sunlight. After 24 h, the clusters were sampled and immediately assayed for ACO activity and juice acidity or stored at -80°C . Berry diameter was measured using calipers as described by Coombe (1992). Titratable acidity of the juice was measured with 0.1 N NaOH up to pH 7. The total anthocyanin content was assayed according to Boss et al. (1996) and converted to malvidin-3-glucoside equivalents using a ϵ of $28,000 \text{ mol}^{-1} \text{ cm}^{-1}$ at 520 nm (Souquet, J.M., pers. comm.).

The internal ethylene was assessed according to Coombe and Hale (1973). Briefly, control whole clusters that had not been incubated in plastic bags, weighing a total of $\sim 150 \text{ g}$, were placed in a bowl filled with a NaCl solution at saturation, under an inverted funnel with an exhaust blocked by a rubber septum. The air remaining in the funnel exhaust was taken out with a syringe. Then the bowl was incubated under a partial vacuum of -700 mm Hg for 5 min using a freeze-dryer chamber. After returning to atmospheric pressure one ml of the internal atmosphere within the funnel directly under the septum was sampled with a syringe and injected in a gas chromatograph.

The *in vivo* ACO activity was assayed using 1 g fresh weight of berry tissue per 1.2 ml of *in vivo* buffer described by Pretel et al. (1995), with the following modifications: Tris-HCl 0.5M, pH 7 and mannitol 0.35 M. The berry content of 1-aminocyclopropane-1-carboxylic acid (ACC) was assayed according to Mansour et al. (1986).

Northern blots were performed according Boss et al. (1996). The ACO cDNA probe was obtained from genomic grape DNA using sequences with GenBank accession number AY211549. The probe matched a 255 bp sequence of the coding region at the 3' end.

A full-length cDNA from *Vitis vinifera* encoding *ETR1* was isolated by screening a lambda ZAP-XR cDNA library constructed from grapevine cell suspension culture mRNA (Loulalakis et al., 1996), using ^{32}P -labelled Arabidopsis *ETR1* C5 as the probe (Chang et al., 1993). DNA in plaque lifts was denatured and fixed according to Sambrook et al. (1989). Filters (Hybond N+, Amersham) were pre-hybridised for 2 h at 50°C in 0.5 M sodium phosphate pH 7.2, containing 7% SDS, 1mM EDTA and 1% BSA and hybridised with cDNA probe for 24 h. Filters were washed under low stringency conditions with 1% SSC and 0.1% SDS at 50°C , and then exposed to X-ray film using intensifying screens, at -70°C for almost 72 h. According to *in vivo* excision properties of lambda ZAP-XR vector, the positive clone was excised as described above. DNA sequencing was performed using a Sequenase DNA sequencing Kit (USB). Sequences were analysed and compared with the GeneBank and EMPL nucleic acid database, using BLAST, BESTFIT and TFASTA programs. The GeneBank accession number is AF243474.

RESULTS AND DISCUSSION

We confirmed the occurrence of an ethylene peak in 'Cabernet Sauvignon' grapes over a three-week period (weeks 6, 7 and 8). This peak represented a concentration of $0.2 \mu\text{L}^{-1}$, which is above the physiological threshold in most plant tissues (Abeles et al., 1992). In addition, *in vivo* activity and transcript accumulation of 1-aminocyclopropane-1-carboxylic acid oxidase (ACO), the last enzyme in the ethylene production pathway, coincided with the occurrence of the ethylene peak (Table 1). The pre-veraison ethylene peak was observed two consecutive years, in irrigated and non-irrigated 'Cabernet Sauvignon' vineyards, and two using different rootstocks ('110R' and '3309C'). It should be pointed out that the timing of the ethylene peak depended on the climatic conditions of the month preceding the peak each year (data not shown).

The total content of ACC, the immediate precursor of ethylene including both conjugated and free forms, reached levels that were 20 times higher than those of the free form alone (Table 1). Therefore, most of the ACC was malonylated and indicates that in grape berries the competition for ACC between ACO and ACC malonyl transferase described previously (Mansour et al., 1986) favours the latter. The levels of total ACC reached approximately 5 nmoles g^{-1} fresh weight at veraison, 1000 times greater than the levels of ethylene production, suggesting that the production of ACC was not limiting.

This high ACC content in grapes had been noted previously (Mizutani et al., 1988). The slight delay between the ethylene peak (week 7) and the ACC peak (week 8) can be explained by the time necessary for the berry tissues to accumulate high levels of ACC. The decrease in ACC levels g^{-1} fresh weight at weeks 9 and 10 can be explained by the resumption of berry growth after veraison (Coombe and McCarthy, 2000).

In order to determine whether this temporary increase in ethylene production has physiological significance in grape ripening, ethylene receptors were blocked with 1-MCP at different times around the expected ethylene peak (i.e. 5 to 9 weeks after anthesis). This molecule has been described as an irreversible inhibitor of ethylene receptors, with an affinity for the receptors 10 times greater than that of ethylene (Blankenship and Dole, 2003). The application of 1-MCP delayed the increase of berry diameter. This delay was correlated to the application of 1-MCP at the time of the ethylene peak. According to Coombe and McCarthy (2000) the beginning of the second growth phase is mainly linked to fluxes within phloem. The roles of ethylene on these fluxes are not well described in the literature. However, ethylene seems to have a role in cell enlargement (Sanchez-Calle et al., 1989; Camp et al., 1981) and this could explain the limitation of diameter increase due to the blockage of ethylene receptors by 1-MCP.

The results also indicate that ethylene may affect the decrease in acid that occurs post-veraison. Grapes treated with 1-MCP at 6, 7 and 8 weeks after anthesis had higher acid levels than the untreated controls when harvested at 13 weeks post-anthesis. The greatest effect of MCP was evident when the treatments coincided with the timing of the endogenous ethylene peak. The decrease in juice acidity at this time is explained mainly by the decrease in malic acid concentration (Ollat et al., 2002). This decrease may be induced by ethylene as an increase in respiration is triggered by this phytohormone even in non-climacteric tissues (Abeles et al., 1992). Indeed, Saulnier-Blache and Bruzeau (1967) showed that several grape cultivars underwent an increase in CO_2 evolution at veraison that could be part of a respiratory burst. It was associated to a lesser extent with a rise in O_2 uptake. This respiratory rise lasted a fortnight following veraison (after which the measurements were stopped) and it seems to match the time in which acidity decreases in the berry. Others have suggested that malic enzyme could also be activated at veraison and be part of malate catabolism (Ollat et al., 2002), and this enzyme has also been shown to be induced by ethylene in ripening fruit (Mamedov et al., 1997). Moreover, the transport of organic acids within cell compartments is obviously involved in acid metabolism (Terrier and Romieu, 2001) and this transport may be modulated by ethylene signals (Schmidt et al., 2003). However, it cannot be ruled out that the sustained acidity could simply result from the inhibition of fruit expansion.

Finally, 1-MCP was shown to transiently inhibit anthocyanin accumulation in berry skins (Table 2). Again this inhibition was greater when 1-MCP was applied at the time of the ethylene peak. This is not surprising as the expression of several enzymes of the anthocyanin pathway (Robinson and Davies, 2000) can be induced by ethylene (El-Kereamy et al., 2003). It is also possible that reduced fruit expansion may have an effect on other signals leading to anthocyanin synthesis and accumulation, i.e. sugar levels (Vitrac et al., 2000). Indeed, it is known that rapid sugar accumulation in berries starts around veraison and is linked to phloem unloading (Coombe and McCarthy, 2000).

Such 1-MCP experiments have been conducted over two consecutive years and similar results have been observed. The results presented here are the data set of a single year, because the time at which the sensitivity to 1-MCP is maximal depends on the climate in the month following bloom, which also impacts the ethylene peak. In these experiments (Table 2), the berries were picked a few weeks before harvest as we noticed in preliminary trials that treated grapes can overcome the 1-MCP inhibition of ripening as time goes by, may be via de novo synthesis of ethylene receptors.

Our observations regarding the role of internal ethylene in modulating several metabolic pathways associated with berry development and ripening, confirm observations of other researchers. Hale et al. (1970) and others (Weaver and Montgomery, 1974; Shulman et al., 1985) observed that these ethylene applications

decreased acidity and enhanced the accumulation of red pigments. This suggests that berry tissues are able to sense ethylene but during the 1970s nothing was known about ethylene signal transduction. Since then, commercial treatments with ethylene precursors have been developed but these precursors are applied at rates that would give rise to an internal concentration of ethylene more than $500 \mu\text{l L}^{-1}$ presuming every mole of the precursor penetrates the plant tissue and is transformed to ethylene. It has been suggested that such treatments result in too high a concentration to have any physiological meaning to the plant in response to this treatment. However, it has been shown such treatments give rise to internal concentrations of ethylene that are 100 times smaller than expected (El-Kereamy et al., 2003).

One could argue that the delay in ripening induced by 1-MCP was due to a toxic effect. However, two arguments against this are: (i) the changes induced by 1-MCP are opposite to those induced by exogenous ethylene application (Weaver and Montgomery, 1974; Shulman et al., 1985); and (ii) the same 1-MCP dose had no effect on berry physiology (i.e. no toxic effect) if applied before or after the ethylene peak.

We found that *VvETR1* transcripts were present during the whole period of berry development up to veraison in Cabernet Sauvignon (data not given). However, there was a slight increase in transcript accumulation over the weeks preceding colour change in most berries. To our knowledge, this is the first expression study of ethylene receptor genes in grapes. It is possible that our cDNA probe picked transcripts related to *ETR1*, as a BLASTN survey (NCBI) with the probe sequence returned homologies with some *ETR2* and *ETR3*, members of the ethylene receptor family, but scores above 100 bits were only for homologies with *ETR1*.

To check whether the transcript changes mentioned above were dependent on the state of the ethylene receptors, we blocked the latter with 1-MCP. We found that the *VvETR1* transcript levels were reduced by the 1-MCP treatment. The mean hybridisation signal in control berries over the period from 6 to 10 weeks after full bloom was 88 (arbitrary units), when it was only 55 for the MCP treated berries. This reduction was significant ($P < 0.001$) and that the difference in the mean values among the different levels of treatment (control or 1-MCP) was greater than would be expected by chance after allowing for effects of differences in date (6, 7, 8, 9 and 10 weeks after full bloom). There was no significant effect of the date ($P = 0.239$). These results indicate that mRNA accumulation of the *VvETR1* gene is dependent on the signalling state of existing receptors (i.e. blocked or not by 1-MCP). To our knowledge this is the first negative feedback ever demonstrated in the ethylene signal perception pathway in grapes and one of the first in plants.

CONCLUSION

It appears that grapes contain a functional network of ethylene signalling at the onset of ripening and part of this complex is necessary to the ripening processes. Our data do not imply that grape should be considered as a climacteric fruit but that new techniques and tools may change the way of categorizing fruit ripening. Further interesting studies are planned, particularly with the development of grape micro-arrays. These studies will bring new insights into the triggering events of ripening in non-climacteric fruit.

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Tables

Table 1. The variation in several parameters related to ethylene production in 'Cabernet Sauvignon' grapes, in comparison to baseline levels, 5 to 10 weeks after anthesis (time is given in weeks after anthesis). ACC = 1-aminocyclopropane-1-carboxylic acid; ACO = ACC oxidase.

	Percent Change	Time at which max levels were measured
Internal ethylene	+ 110%	week 7
ACO RNA accumulation	+ 400%	week 7
ACO activity	+ 100%	week 7
Total ACC	+ 130%	week 8
Free ACC	no change	-

Table 2. Variation in three grape maturity parameters of 'Cabernet Sauvignon' berries harvested 13 weeks after full bloom. The fruit had been gassed for 24 hours with 4 ppm MCP at various times (5 to 9 weeks) after full bloom. All variations were significant at the 5% level.

	Maximum variation in comparison to the control levels*	Application time at which the MCP treatment gave rise to maximum variation
Berry diameter	- 15%	week 7
Total acidity	+ 20%	week 7
Total anthocyanins	- 60%	week 8

Analyses of variance were performed with SigmaStat (SPSS Inc., Chicago, IL) and LSDs at the 0.05 level determined.