

Insight

Key enzymes behind black pepper aroma in wines

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Sesquiterpene rotundone provides aroma in common herbs such as rosemary and thyme, and is responsible for the peppery scent of Shiraz wines. In this issue of *Journal of Experimental Botany*, Takase *et al.* (pages 787–798) and Drew *et al.* (pages 799–808) provide the basis for understanding the key steps leading to the synthesis of alpha-guaiene and its enzymatic oxidation to rotundone in the grapevine berry at a molecular level.

Humans have developed a sense of smell based on about 400 olfactory receptors, each capable of selectively detecting individual molecules or small groups of substances with well-defined structural requirements. It has been suggested that 230 key molecules can reconstruct the aroma of 220

food samples (Dunkel *et al.*, 2014). However, explaining wine aroma is far more complex (Box 1). The volatiles measurable in a wine can exceed 1500, and compounds present at trace levels are often key odorants. The discovery of genetic factors governing the complexity of wine aroma is therefore a challenging but important goal, with expected applications in viticulture, oenology and other fields.

One of the most intriguing and characteristic aromas in wine is the spicy-peppery note, only found in grapes and wines produced using selected cultivars. It is a common descriptor for wines produced from red cultivars such as Syrah, also known as Shiraz in Australia, as well as Cagnulari, Schioppettino, Vespolina and several others in Italy, Graciano in Spain, and the white cultivar Gruene Veltliner, the flagship of Austrian oenology.

Box 1. Explaining the aroma of wine is incredibly complex. Let us start with our vocabulary for flavourings, based on prototypes. We are used to classifying any aroma by its similarity to a reference, a subjective operation closely dependent on individual experience and training. As a result, in everyday life, the scent of a rose is described as ‘from rose’, that of bell pepper ‘herbaceous, from pepper’, and the same happens for the scent of carnations, passion fruit, saffron, black pepper, etc. They can vary in intensity and refinement (for example, the damask rose is rated more highly than other roses) but the perfume can almost always be linked to the expected prototype. This is not the case for wine, resulting from fermentation of the fruit of a highly heterozygous species producing innumerable chemotypes. As a consequence of this genetic diversity, further multiplied by the generation of multiple compounds following fermentation and chemical ageing, different wines can display an incredible array of distinctive aromas. Image courtesy of Archive Fondazione Edmund Mach – M. Brega.



Elusive rotundone

It is surprising that the key aroma of pepper, the most important spice traded internationally, was only discovered by chemists investigating the aroma of Shiraz wine – but it is highly elusive. When rotundone was synthesized in our lab (Mattivi *et al.*, 2011), to our disappointment the tube containing a bulk amount of the pure compound dissolved in pentane was found to have a kind of balsamic scent, in no way reminiscent of the spice. Only afterwards, when the precious liquid had been transferred for safe storage, did we observe with relief that the rinsed glassware, containing only traces of rotundone and left to dry under a chemical hood to eliminate solvents, was capable of filling the room with the desired intense smell of black pepper.

As highlighted in the review by Schwab and Wuest (2015), one of the most surprising results of recent studies on the origin of terpenoid odorants (monoterpenes and sesquiterpenes) in wine is the mixed formation mechanisms encompassing enzymatic and non-enzymatic steps. The formation of rotundone has been postulated to require the enzymatic formation of the precursor alpha-guaiene, followed by non-enzymatic formation of rotundone by aerial autoxidation of alpha-guaiene.

The paper by Takase *et al.* (2016) offers additional information in the quest to understand the origin of rotundone in grapes. The Japanese team identified a new cytochrome P450 enzyme, named VvSTO2, generating gene-specific primers from the information obtained via screening with the homology sequence of known sesquiterpene oxidase against the 12-fold coverage genome sequence assembly of the PN40024 genotype (Jaillon *et al.*, 2007). Using PCR with specific primers, VvSTO2, an enzyme classified as CYP71BE5, was then isolated from the exocarp of the grapevine, cv. Syrah. Functional characterization revealed that VvSTO2 is a guaiene 2-oxidase, capable of oxidizing the C2 position of alpha-guaiene, leading to the biosynthesis of (–)-rotundone as a major reaction product. This enzyme has limited specificity, accepting alpha-guaiene as a substrate. This study suggests that the accumulation of rotundone is dependent on both the biosynthesis of alpha-guaiene and the expression of the key enzyme VvSTO2.

Rotundone's direct precursor

After gaining this new insight, the next biochemical question is to understand why certain grape cultivars such as Syrah accumulate the direct precursor of rotundone, sesquiterpene alpha-guaiene – a difficult task, considering that as many as 69 terpene synthases (TPSs) were identified in grapevine using analysis of the 12-fold coverage genome sequence assembly of the PN40024 grapevine (Schwab and Wuest, 2015).

This intriguing question is answered in research by Drew *et al.* (2016). These authors were able to isolate the new gene *VvGuaS*, a polymorphic variant of the known *VvTPS24* gene, from Shiraz berries. The gene was shown to synthesize a mixture of alpha-guaiene and delta-guaiene as main products when expressed in *Nicotiana benthamiana* leaves. Interestingly, no sequences from the PN40024 genotype

genome matched the *VvGuaS* variant. This reference genome contained only the *VvTPS24* gene encoding VvPNSeInt, an enzyme previously characterized and found to produce other sesquiterpenes. However, Drew *et al.* reported that four out of the six variants in the amino acid sequences of VvGuaS in respect of VvPNSeInt, including those at the 414, 431, 499 and 530 positions they were searching for, were the predicted protein product of contig VV78X107636.8, contained in the Pinot Noir genome sequence of Velasco *et al.* (2007). Detailed functional analysis of the VvGuaS enzyme revealed that a change in just two amino acids out of 561 is capable of dramatically changing the function of the enzyme, leading to the production of alpha-guaiene.

It was shown that a two amino acid residue polymorphism at the active site of the protein (T414S/V530M double mutation) suppressed the ability of wild-type protein to produce alpha-guaiene – a striking example of a tiny mutation conferring a new function. Muscat aroma in grapes, discovered via QTL analysis followed by a candidate gene approach (Battilana *et al.*, 2009, 2011), is similarly dramatic. The floral aroma of the large family of Muscat-related grape cultivars is due to a single nucleotide mutation in the *VvDXS* gene (Emanuelli *et al.*, 2010) and represents one of the few human-selected qualitative traits, a gain in terms of function/products, leading to the presence of several floral terpenes in *Vitis vinifera* grapes that are absent in other species (Narduzzi *et al.*, 2015).

It is worth highlighting here that analysis of the genome of the highly inbred Pinot Noir PN40024 could not reveal the presence of the *VvGuaS* variant, since it is a homozygote for *VvPNSeInt*. In contrast, in the highly heterozygous Pinot Noir genome (Velasco *et al.*, 2007) a terpene synthase gene, 97.5% homologous in encoded amino acids to the gene in PN40024, can be found, in heterozygosis, with the presence of the two amino acids required to confer the function of alpha-guaiene production in one of the two alleles. In conclusion, both alleles of terpene synthase reported as *VvGuaS* and *VvPNSeInt* are present only in 'real' Pinot Noir. Moreover, on the same chromosome (19) of Pinot Noir, it is possible to observe the presence 'in tandem' of a second isoform of a similar gene with 93.1% homology, in homozygosis, but with different amino acids in the critical positions 414 and 530.

What next?

With this new knowledge, immediate progress in understanding the genetic factors leading to the variable production of rotundone in grapes can be expected through future analysis of the alleles in several 'spicy' cultivars, paving the way for the breeding of new, spicy grape cultivars. Likewise other recent papers which have discovered the function of transcription factors controlling quantitative variation of important traits in grapevine (Fournier-Level *et al.*, 2009; Ocares and Mejia, 2015). Today more than ever genetics and breeding are intimately interconnected.

Both papers (Drew *et al.*, 2016; Takase *et al.*, 2016) investigated candidate genes based on putative functions, leaving the field open for investigating which gene(s) actually control the final accumulation of rotundone in the grapevine berry. It is well

known that the expression of rotundone in the berry is highly dependent on environmental parameters, with cooler seasons leading to much higher accumulation and a high degree of variability observed within the same vineyard (Caputi *et al.*, 2011). Further investigations of environment-dependent enzymes and transcription factors promoting the accumulation of rotundone in the berry are needed to complete the picture. I hope that the globally recognised teams of researchers carrying out these brilliant experiments can make rapid progress, inspiring others to use our new biochemical understanding for comparative studies of the origin of rotundone among common herbs and spices, such as basil, marjoram, oregano, rosemary and thyme (Wood *et al.*, 2008). From spicy wine back to spices proper.

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Insight

How can we breed for more water use-efficient sugarcane?

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Selection on the basis of physiological traits is hedged with obstacles in conventional breeding programmes – it is a little-explored concept. However, in this issue of *Journal of Experimental Botany* (pages 861–872), Jackson *et al.* present research in which the broad-sense heritability of leaf- and crop-level transpiration efficiency was tested within the framework of Australia’s main sugarcane breeding programme.

Conventional breeding mostly consists of large-scale crosses followed by quick selection methods. To date, most breeding programmes do not use physiological indices, while some rely on experienced breeders walking through field or nursery trials and visually selecting the winners for the following stages. Further, breeders mostly select for vigour and disease resistance. Therefore, selecting for physiological