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A grapevine cytochrome P450 generates the precursor of wine lactone, a key odorant in wine

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Summary

- Monoterpenes are important constituents of the aromas of food and beverages, including wine. Among monoterpenes in wines, wine lactone has the most potent odor. It was proposed to form *via* acid-catalyzed cyclization of (*E*)-8-carboxylinalool during wine maturation. It only reaches very low concentrations in wine but its extremely low odor detection threshold makes it an important aroma compound.
- Using LC-MS/MS, we show here that the (*E*)-8-carboxylinalool content in wines correlates with their wine lactone content and estimate the kinetic constant for the very slow formation of wine lactone from (*E*)-8-carboxylinalool. We show that (*E*)-8-carboxylinalool is accumulated as a glycoside in grape (*Vitis vinifera*) berries and that one of the cytochrome P450 enzymes most highly expressed in maturing berries, CYP76F14, efficiently oxidizes linalool to (*E*)-8-carboxylinalool.
- Our analysis of (*E*)-8-carboxylinalool in Riesling × Gewurztraminer grapevine progeny established that the *CYP76F14* gene co-locates with a quantitative trait locus for (*E*)-8-carboxylinalool content in grape berries.
- Our data support the role of CYP76F14 as the major (*E*)-8-carboxylinalool synthase in grape berries and the role of (*E*)-8-carboxylinalool as a precursor to wine lactone in wine, providing new insights into wine and grape aroma metabolism, and new methods for food and aroma research and production.

Introduction

Grapevine (*Vitis vinifera*) is one of the most economically important crops worldwide with most of the annual 70 million tons of grape harvest used for winemaking (http://www.oiv.int/). The quality of wine depends to a large extent on its aroma. However, the chemical complexity of wine aroma makes it a difficult subject to study, let alone predict: it consists of several hundred different volatile compounds at concentrations spanning several orders of magnitude (Francis & Newton, 2005). The advances in analytical chemistry in the second half of the 20th century allowed scientists to study this complex chemical mixture in more detail. It gradually transpired that a characteristic wine aroma does not depend as much on the most abundant compounds as on trace components with very strong odors (Francis & Newton, 2005). One is isobutyl methoxypyrazine, responsible for the characteristic green (bell) pepper scent of Cabernet Sauvignon and Sauvignon Blanc wines, where it reaches concentrations of *c.* 30 pg l^{-1} (Harris *et al.*, 1987). Another example is rotundone, a sesquiterpene which conveys the peppery aroma to Shiraz wines at concentrations as low as 20 ng l^{-1} (Wood *et al.*, 2008). Wine lactone, a bicyclic monoterpene lactone, is another trace aroma compound in wines with a very low odor threshold (10 ng l^{-1}) (Guth, 1997b). Pure wine lactone has a sweet, woody and coconut-like aroma (Guth, 1996), and was shown to contribute to the aroma of Gewurztraminer wines (Guth, 1997a,b). It might also contribute to the aroma profile of fresh orange (Hinterholzer & Schieberle, 1998) and grapefruit (Buettner & Schieberle, 1999) juices.

As opposed to rotundone or isobutyl methoxypyrazine, wine lactone is not produced in grapes, but predominantly during wine maturation (Guth, 1998). It forms in a slow, nonenzymatic, acid-catalyzed cyclisation from an odorless precursor, (*E*)-8-carboxylinalool (also known as menthiafolic acid; Bonnländer *et al.*, 1998; Luan *et al.*, 2006; Giaccio *et al.*, 2011), and it has been suggested that wine lactone concentration increases as wine ages (Giaccio *et al.*, 2011). However, the data on both (*E*)-8-carboxylinalool and wine lactone concentrations in wine are scarce. Concentrations of wine lactone in wine are very low, and thus its quantification requires tedious sample preparation (Giaccio *et al.*, 2015). (*E*)-8-carboxylinalool is often overlooked because only gas chromatography is used for the analysis of

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aroma components, although it is not appropriate for the detection of polar compounds. A more extensive analysis is additionally impeded by the fact that (*E*)-8-carboxylinalool is often found, both in grapes and wines, as a glucose ester with the sugar moiety attached to the carboxyl functional group (Winterhalter *et al.*, 1997; Bonnländer *et al.*, 1998; D'Ambrosio *et al.*, 2013). Monoterpene glycosides and glucose esters were suggested to act as a precursor pool for the formation of aroma compounds during wine fermentation and maturation, when they are hydrolyzed enzymatically or chemically (at low pH) (Williams *et al.*, 1980; Schwab & Wüst, 2015).

Luan *et al.* (2004) proposed that (*E*)-8-carboxylinalool is formed in grapes from the monoterpene linalool (Luan *et al.*, 2004), another important aroma compound, in a multi-step enzymatic reaction: linalool is first oxidized at C8 to yield (*E*)-8-hydroxylinalool, also a common grape metabolite (Strauss *et al.*, 1988), which is then further oxidized at the same carbon atom to (*E*)-8-carboxylinalool (Fig. 1). This precursor can be glycosylated to form the glucose ester. Both free and bound (*E*)-8-carboxylinalool were suggested to act as precursors to wine lactone in a slow cyclisation reaction that takes place upon wine maturation (Giaccio *et al.*, 2011).

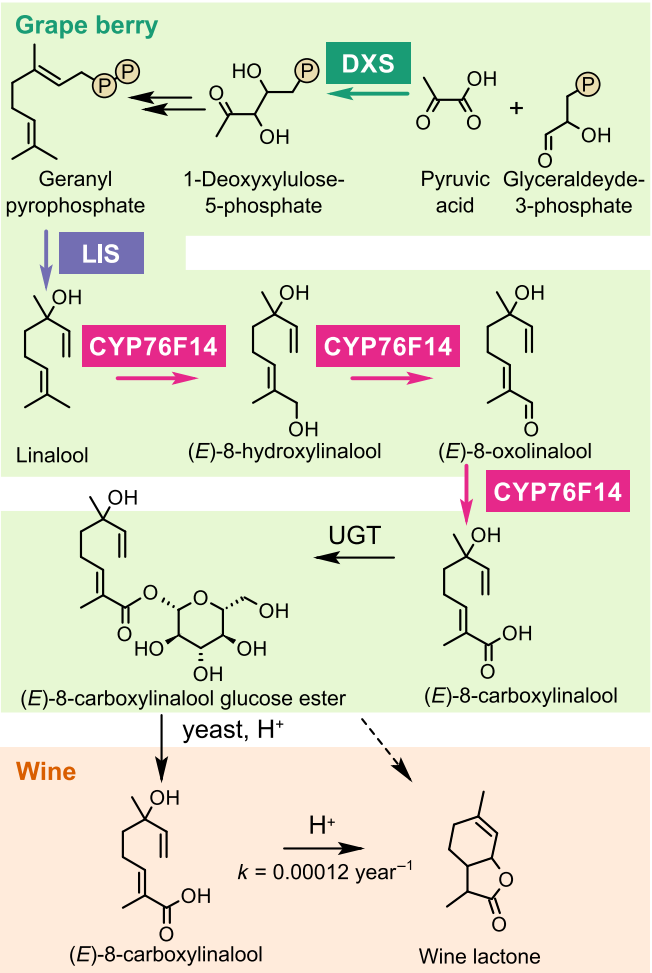

Fig. 1 The pathway of wine lactone formation. Highlighted enzymes (DXS, 1-deoxy-D-xylulose-5-phosphate synthase; LIS, linalool synthase, CYP76F14) correspond to the quantitative trait loci (QTLs) associated with grape berries (*E*)-8-carboxylinalool concentration. [Correction added after online publication 25 August 2016: in the reaction pathway products (*E*)-8-hydroxylinalool and (*E*)-8-oxolinalool have been corrected.]

Several terpene synthases (TPS) from grapevine have been reported to generate linalool (Martin *et al.*, 2010), which *per se* constitutes an important bouquet component of the so-called aromatic white wines such as Muscat, Riesling and Gewurztraminer. There is no record of enzymes further processing linalool in grape, but recent work describes cytochrome P450-dependent cascade oxidations of monoterpenols such as linalool in *Arabidopsis thaliana* (Ginglinger *et al.*, 2013; Hofer *et al.*, 2014; Boachon *et al.*, 2015) or geraniol in *Catharanthus roseus* (Collu *et al.*, 2001).

In the present study, we explore the pathway of wine lactone formation, from the early enzymatic steps during grape berry development, to the nonenzymatic steps during wine maturation and aging. Using liquid chromatography coupled to mass spectrometry, we first establish the relationship between the concentrations of wine lactone and its precursors in a selection of wines of different ages. We show that the expression of a subset of P450 genes is induced in the late stages of berry ripening and demonstrate that, among these, only CYP76F14, which is the most highly expressed CYP76 in ripe berries, efficiently catalyzes the conversion of linalool to (*E*)-8-carboxylinalool both *in vitro* and *in planta*. The *CYP76F14* gene maps to one of the three QTLs associated with grape berry (*E*)-8-carboxylinalool content. We therefore propose that CYP76F14 is responsible for the synthesis of (*E*)-8-carboxylinalool, the wine lactone precursor, in grapes.

Materials and Methods

Synthesis of monoterpenoids

(*E*)-8-hydroxylinalool, (*E*)-8-oxolinalool and (*E*)-8-carboxylinalool were synthesized and purified as described previously (Ginglinger *et al.*, 2013; Boachon *et al.*, 2015). Enantiomerically pure wine lactone was synthesized according to the previously described procedure (Chavan *et al.*, 2001) with the following modifications: hydroboration was accomplished following the previously described procedure (Bode & Carreira, 2001) and separation of the diastereomers was carried out after the dehydrohalogenation step, contrary to recommendation (Chavan *et al.*, 2001). Wine lactone displayed identical spectroscopic data to those reported previously.

Wine samples

Wines were purchased from Paul Ginglinger (Eguisheim, France) or produced by the INRA Colmar (France). One sample (Riesling Weingut 2014) was purchased at the supermarket. The INRA Gewurztraminer wines were made from grapes collected from the same vineyard localized in the 'Grand Cru Osterberg' area in Ribeauvillé (France) in 1986, 1997, 1998, 2001, 2004, 2005, 2007 and 2009. All wines were stored in the INRA cellar, which is maintained at the constant temperature of 10°C. For

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the analysis of (*E*)-8-carboxylinalool and (*E*)-8-carboxylinalool glucose ester, wines were injected directly to LC-MS/MS without being extracted or concentrated. For the analysis of wine lactone, 40 ml of wine was spiked with internal standard ((*E*)-8-oxolinalool), then extracted with 10 ml pentane: ethyl acetate (1 : 1 v/v). The extract was then evaporated to dryness and the residue resuspended in 200 µl of methanol before LC-MS/MS analysis.

Liquid chromatography coupled to tandem mass spectrometry

Liquid chromatography-mass spectrometry analyses were performed on a Waters Quattro Premier XE mass spectrometer equipped with an electrospray ionization source and coupled to an Acquity UPLC system (Waters, Milford, MA, USA). Chromatographic separation was achieved using an Acquity UPLC bridged ethyl hybrid C18 column (100 × 3 × 2.1 mm, 1.7 µm; Waters). The mobile phase consisted of (A) water and (B) methanol, both containing 0.1% formic acid. The solvent gradient was as follows: 95% A 0–2 min, linear gradient to 0% A 2–12 min, 0% A 12–14 min, linear gradient to 95% A 14–15 min, 95% A 15–17 min. The flow rate was 0.350 ml min^{-1}. The column was heated to 35°C. Injection volume was 5 µl for multiple reaction monitoring (MRM) runs and 3 µl for nontargeted (scan) runs. Nitrogen was used as the drying and nebulizing gas. The nebulizer gas flow was set to 50 l h^{-1}, and the desolvation gas flow to 900 l h^{-1}. The interface temperature was 400°C and the source temperature 135°C. The capillary voltage was set to 3.4 kV; for the scan runs the cone voltage was set to 25 V and the ionization was in positive or negative mode. Low mass and high mass resolution was 15 for both mass analyzers, ion energies 1 and 2 were 0.5 V, entrance and exit potential were 50 V, and detector (multiplier) gain was 650 V. Parent and daughter ions used for MRM were: 137 > 80.7 for linalool, 135 > 106.8 for (*E*)-8-hydroxylinalool, 151.2 > 92.8 for (*E*)-8-oxolinalool, 167.2 > 92.8 for (*E*)-8-carboxylinalool and 167.2 > 93 for wine lactone. Glycosylated derivatives were putatively identified on the basis of their mass spectra (Supporting Information Table S1). Statistical analysis, kinetic modeling and data visualization were performed using R software (R v.3.2.2; R Core Team, 2013).

For estimation of the kinetic constant, data were fitted to the following equation,

$$[\text{Wine lactone}] = (1 - e^{-k \cdot \text{Wineage}}) \times [(E) - 8 - \text{carboxylinalool}]$$

using SD^{-2} as weights.

Selection of candidate genes

Sequences of cytochrome P450 genes in the *Vitis vinifera* L. PN40024 genome were retrieved from the Grape genome database (http://genomes.cribi.unipd.it/grape/) and their translated sequences were aligned to the P450 sequences from the heterozygous Pinot noir genome from the cytochrome P450 website (http://drnelson.uthsc.edu/CytochromeP450.html). In cases of discrepancy, the genes were manually re-annotated. RNA sequencing data from Riesling and Gewurztraminer grape berries from two time points (green and mid-ripe) were aligned to these gene models. The RNA sequencing experiment will be reported elsewhere. For further investigations, we chose those genes with increased expression at the later time point (mid-ripe): CYP71AT7 (gi|731429818|ref|XM_002278264.3|, CYP76F12: gi|731379818|ref|XM_010663319.1|, CYP76F14: gi|731377631|ref|XM_010659734.1|, CYP76T21: gi|731421027|ref|XM_002276540.3|, CYP76Y1: gi|731384210|ref|XM_010649735.1|, CYP76Y2: gi|731380436|ref|XM_003631420.2|).

Total RNA isolation from grape berries and qPCR

Grape berries were collected from the Gewurztraminer 643 clone from the ampelographic collection of INRA Colmar, at five different developmental stages: green (4 July 2014), before and after veraison (8 August 2014), mid-ripe (8 September 2014) and ripe (29 September 2014). Berries were flash frozen in liquid nitrogen and stored at −80°C. They were crushed with a hammer to allow the removal of seeds. Then, they were ground using a Qiagen Teflon grinding jar set and TissueLyser for 30 s at 30 s^{-1}.

Total RNA was isolated from 1 g of berry powder according to the described procedure (Reid *et al.*, 2006) with the following modifications: 7 ml of extraction buffer was used per gram of berry powder and after LiCl precipitation the samples were treated as described above. cDNA was synthesized from 1 µg total RNA using $(dT)_{23}$ and Superscript® III Reverse Transcriptase (Life Technologies, Carlsbad, CA, USA) following the manufacturer's instructions. The resulting cDNA was diluted 5× and used as a template in quantitative PCR (qPCR) experiments.

Primers for qPCR were designed using the Primer3 plus website and are listed in Table S2. Specificity of the primers was confirmed *in silico* using the Primer Blast website (NCBI) and by measuring the melting curve of the products. The reaction mixture contained 1× SYBR® Green Master Mix, 250 nM of forward and reverse primers and 50× diluted cDNA from reverse transcription reaction. The qPCR experiment was performed on a Roche LightCycler® 480. The three reference genes, EF1-α (XM_002284928.3), UBQ_L40 (XM_002273532.2) and MDH (m) (XM_002278676.3), were selected from six constitutively expressed grapevine genes (Reid *et al.*, 2006) using the GeNorm algorithm (Vandesompele *et al.*, 2002). Amplification efficiency was calculated for each set of primers using LinRegPCR (Ramakers *et al.*, 2003). Relative gene expression was calculated as described previously (Hilliou & Tran, 2013). Each point was obtained from three technical and three biological replicates.

Amplification of coding sequences, construction of vectors and recombinant expression

RNA was extracted from grape skins of *V. vinifera* cv Muscat Ottonel following a previously described procedure (Reid *et al.*, 2006), except that PVPP in the extraction buffer was replaced

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with PVP. After LiCl precipitation and ethanol wash, the nucleic acids were digested using RQ1 RNase-Free DNase (Promega, cat. no. M6101). The reaction mixture was diluted to 250 µl and extracted with an equal volume of phenol : chloroform : isoamyl alcohol (25 : 24 : 1 v/v). The aqueous phase was extracted twice with an equal volume of chloroform. RNA was precipitated with ethanol and resuspended in 30 µl of MilliQ water. cDNA was synthesized using Superscript® III Reverse Transcriptase (Life Technologies) following the manufacturer's instructions, and was then used as a template for PCR amplification of the target sequences.

CYP76F14 and CYP76F12 PCR products were cloned into the pYeDP60 yeast expression vector using restriction enzymes Bam*HI* and Kpn*I*. The N-terminal segment of CYP76F14 was recoded taking into account yeast codon preference using a 150-nt forward primer. The PCR product was first inserted into pGEM®-T Easy vector (Promega) before being transferred to pYeDP60 using the same restriction enzymes. CYP71AT7, CYP76T21, CYP76Y1 and CYP76Y2 were inserted into the yeast expression vector pYeDP60u2 using the USER™ cloning method (Nour-Eldin *et al.*, 2006). Gene sequences were verified by sequencing. Muscat Ottonel was used for sequence amplification and some sequence divergence with the reference genome (PN40024) was expected. In the cases where sequence divergences were consistent in several colonies, they were considered as relevant and the corresponding amplicons were expressed in yeast. The PN40024 CYP76F14 coding sequence was synthesized *de novo* by Genecust Europe (Dudelange, Luxembourg) with codon optimization for expression in yeast. CYP76F12, CYP76F14 and CYP76T21 were cloned into the pCAMBIA2300 vector with the USER™ cloning method. All cloning primers sequences are listed in Table S3. Sequences described in this work have been deposited in GenBank under accession numbers KU955332 to KU955337.

cDNA sequences of putative LIS expressed in Gewurztraminer berries were reconstituted based on RNAseq data and on homology with LIS identified in the grapevine reference genome (Martin *et al.*, 2010). A full-length cDNA corresponding to a putative TPS56 (HM807392 PN55M1) was then amplified from RNAs prepared from mid-ripe Gewurztraminer berries and cloned into vector pMDC32.

Heterologous expression in yeast

The *Saccharomyces cerevisiae* strain WAT11 was transformed with pYeDP60:CYP or 'empty' pYeDP60 plasmid as described previously (Gietz *et al.*, 1992). Yeast cultures were grown as described for the high density procedure (Pompon *et al.*, 1996) with the following modifications. A 200-ml culture volume was inoculated with 15 ml of the overnight culture, and gene expression was induced by the addition of 5% vol. of 200 g l^{-1} galactose. The microsomal membranes were isolated as described previously (Gavira *et al.*, 2013). The expression of P450 was evaluated by CO differential spectroscopy of reduced microsomes (Omura & Sato, 1964). The intensity of the peak at 450 nm was too low to allow for reliable quantification of enzymes.

P450 enzyme assays

In vitro P450 enzyme assays were conducted in 20 mM Na^+/K^+ phosphate buffer, pH 7.4, in the presence of 730 µM reduced NADPH, 100 µM of substrate and 10% vol. of microsomal membrane suspension. Reactions were conducted at 27°C for 1 h under agitation and were stopped by the addition of solvent (ethyl acetate for samples to be analyzed by GC or methanol for samples to be analyzed by LC), vortexed for 30 s and centrifuged to pellet the membranes and proteins. Ethyl acetate extracts were filtered over sodium sulfate to remove residual water. All assays were performed in triplicates.

Transient expression in *N. benthamiana*

Nicotiana benthamiana plants were grown under 16 h (24°C) : 8 h (20°C), day : night conditions. The *Agrobacterium tumefaciens* strain LBA4404 was transformed with the pCambia2300 or pCambia3300 plasmids by electroporation. Liquid cultures of transformants (100 ml LB, 50 µM rifampicin, 10 µM gentamicin, 50 µM kanamycin) were grown overnight at 28°C (180 rpm agitation). The next day they were harvested by 10 min centrifugation at 3000 ***g*** and washed twice with tap water. Agrobacteria transformed with the p19 gene of tomato bushy stunt virus (Qu & Morris, 2002), linalool synthase and P450 genes were then mixed with tap water in the same tube in 1 : 1 : 1 ratios, so that the OD_{600} of each of the strains corresponded to 0.4. The bacteria then were infiltrated into the abaxial epidermis of 20-d-old *N. benthamiana* leaves with a 1-ml plastic syringe (without needle). For negative controls, strains for expression of either P450 or LIS were replaced with a GFP expression strain.

Leaf tissues were collected 4 d post-infiltration, frozen in liquid nitrogen and ground with a metal bead (7 or 10 mm diameter) in 12-ml plastic tubes. Leaves expressing GFP were also viewed under UV light to confirm successful agroinfiltration and gene expression. Leaf powder was stored at −80°C and extracted by vortexing with methanol (1 : 4 m/v). Extracts were then stored overnight at −20°C and centrifuged just before analysis.

Gas chromatography analysis

Ethyl acetate extracts were analyzed on a Varian 3900 gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) with flame ionization detector, equipped with a DB5 column (Agilent Technologies). Injector temperature was 250°C and injection was splitless. Temperature gradient was: 0.5 min at 50°C, 10°C min^{-1} to 320°C, and 5 min at 320°C. Temperature of the FID detector was 280°C.

Experimental conditions and sampling for QTL analysis

For the detection of quantitative trait loci (QTL) for the (*E*)-8-carboxylinalool concentration in grapevine berries, a grapevine population consisting of the progeny from a cross between

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V. vinifera cv Riesling (Ri) clone 49 (female) and *V. vinifera* cv Gewurztraminer (Gw) clone 643 (male) (Ri × Gw) was used. 383 genotypes from the Ri × Gw cross were grafted onto the Couderc 161-49 rootstock and three plants per genotype were planted in an experimental vineyard in Bergheim (48°21′ N, 7°34′ E) in 2006. The veraison dates (onset of the ripening process) were calculated for each individual plot, after successive scorings, as the dates when 50% of the berries were soft (entering the ripening process).

One hundred millilitres of berries were collected from 120 genotypes during ripening. The sampling date was determined for each genotype as the date when a cumulative heat sum of 350 degree-days (calculated with maximum temperatures, base temperature = 10°C) after veraison was reached. This procedure was validated previously for assessing genotypic differences for sugar accumulation capacity (Duchêne *et al.*, 2012b) and berry acidity (Duchêne *et al.*, 2014). Some of the berries were used for sugar and acid analysis and a sample was frozen at −20°C before HPLC analysis.

UHPLC analysis of (*E*)-8-carboxylinalool in Ri × Gw progeny

For UHPLC analysis, 40 g of thawed berries were deseeded and crushed under liquid nitrogen. The powder was suspended in a 1 g l^{-1} Na_2SO_3 solution. After centrifugation (25 min, 16 500 ***g*** at 4°C), the supernatant was passed through a glass fiber prefilter and a glass filter; 150 µl of a 100 g l^{-1} apigenin solution was added as external standard. The filtrate was passed through a 1 g C18 silica-bonded nonpolar solid phase extraction (SPE) cartridge (Bond Elut Jr., Agilent technologies), rinsed previously with 5 ml of methanol and 15 ml of ultrapure water, at a rate of *c.* 1 drop s^{-1}. After washing with 15 ml of ultrapure water, compounds of interest were eluted with 4 ml of ethanol and analyzed by ultra-high performance liquid chromatography and high-resolution mass spectrometry (UHPLC-HRMS).

Acetonitrile and formic acid of liquid chromatography–mass spectrometry grade were supplied by Thermo Fisher. The analysis of methanol extracts was performed using an UHPLC system (Dionex Ultimate 3000; Thermo Fisher Scientific, San Jose, CA, USA) equipped with a diode array detector (DAD). The chromatographic separation was performed on a Nucleodur HTec column (150 × 2 mm, 1.8-µm particle size; Macherey-Nagel, Düren, Germany) maintained at 30°C. The mobile phase consisted of acetonitrile/formic acid (0.1%, v/v) (eluant A) and water/formic acid (0.1%, v/v) (eluant B) at a flow rate of 0.25 ml min^{-1}. The gradient elution program was as follows: 0–4 min, 80–70% B; 4–5 min, 70–50% B; 5–6.5 min, 50% B; 6.5–8.5 min 50–0% B; 8.5–10 min 0% B. The sample volume injected was 1 µl. The liquid chromatography system was coupled to an Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific) equipped with an electrospray ionization source operating in positive mode. Parameters were set at 300°C for ion transfer capillary temperature and 3400 V for needle voltages. Nebulization with nitrogen sheath gas and auxiliary gas were maintained at 40 and 5 arbitrary units, respectively. The spectra were acquired within the *m/z* mass range of 95–1200 atomic mass units (a.m.u.), using a resolution of 50 000 at *m/z* 200 a.m.u. The system was calibrated internally using dibutyl phthalate as lock mass (*m/z* 279.1591), giving a mass accuracy below 1 ppm. Peak integration was performed through the XCALIBUR software and, for compounds of interest, integration was checked manually before validation. In most samples, the ion current corresponding to (*E*)-8-carboxylinalool was complex, suggesting that in addition to its free form, it was also accumulated as multiple glycosylated derivatives, according to their mass and fragmentation patterns. In order to take the full diversity of these compounds into account, the whole carboxylinalool ion current was integrated, to include both the signal corresponding to free carboxylinalool and that coming from fragmentation the glycosylated derivatives. The hypothesis of normality was not satisfied for the quantified signal at the 5% level, even after a $\log_{10}$ transformation (Shapiro–Wilk normality test, *P*-value = 0.009). Nevertheless, the $\log_{10}$-transformed data were kept for further QTL detection analysis, as their distribution was much closer to a normal distribution than the raw data.

QTL detection was performed on a consensus genetic map used previously for the detection of QTLs for developmental stages (Duchêne *et al.*, 2012a). Two genetic markers were added to the initial map. VVsc271 is a simple sequence repeat (SSR) on scaffold 271 in the PN40024 12× reference genome (http://www.genoscope.cns.fr/cgi-bin/ggb/vitis/12X/gbrowse/vitis). Primers used to amplify this region were VVsc271f : 5′-CTTTCATGACAGATTGAGATTTACAACAAG-3′ and VVsc271r : 5′-TAGCTCTTATGACAACTTGAAAATGGAGCC-3′. Scaffold 271 was chosen because several terpene synthases were annotated in this region (Martin *et al.*, 2010).

The *DXS1* gene (GSVIVT00017832001) was mapped by 'High Resolution Melting Assay' (Wittwer *et al.*, 2003) for a [A/C] Single Nucleotide Polymorphism (SNP) at position 4789 in the Gw*DXS1* gene sequence (Duchêne *et al.*, 2009). The final map was built with 153 simple sequence repeats markers (SSR) and a SNP for the *DXS1* gene and covers 1 131.1 cM along the 19 chromosomes of the grapevine genome.

Genetic map

QTL detection was performed on the Ri × Gw consensus map (Duchêne *et al.*, 2012a) with the R/qtl software (Broman *et al.*, 2003) using the multiple imputation method ('draws' = 516) and the one-dimension scan command 'scanone'. LOD significances were ensured with permutation tests (1000 permutations). QTL models were constructed step-by-step after the refinement of the QTL position (refineqtl), the search for supplementary QTLs (addqtl). The LOD score and the percentage of variance explained by a QTL in a QTL model was assessed with analysis of variance using type III sums of squares (fitqtl). Confidence intervals were calculated as Bayesian credible intervals (bayesesint) with a probability of

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coverage of 0.95. The physical positions of our confidence intervals were assessed according to the physical positions of the microsatellite markers in the genome and to their position in our consensus map.

Results

Wine lactone concentration is determined by wine age and precursor concentration

Due to its low abundance, only a few successful attempts at wine lactone quantification in wine have been reported (Guth, 1997a; Giaccio *et al.*, 2015). Our first attempts using gas chromatography coupled to mass spectrometry (GC-MS) revealed that its limit of detection was above the concentrations expected in wines. We then developed a LC-MS/MS method, which enabled the quantification of wine lactone in all the 23 white wines investigated. Although the concentrations of wine lactone detected were low (in the nM range), they exceeded the sensory threshold of wine lactone in all samples, which is 60 pM in model wine (Guth, 1997b). We also used LC-MS/MS to analyze (*E*)-8-carboxylinalool by direct injection of wines without extraction (Fig. 2). With this method, we were able to detect both free (*E*)-8-carboxylinalool and its conjugated form, putatively identified as (*E*)-8-carboxylinalool glucose ester.

In order to determine the factors controlling wine lactone concentrations in wine, we analyzed wines of different ages and varieties for their content of wine lactone and its two precursors, (*E*)-8-carboxylinalool and (*E*)-8-carboxylinalool glucose ester. We observed that wine lactone concentrations increased with wine age, as well as with concentration of (*E*)-8-carboxylinalool (Fig. 2). We used the first-order reaction rate law to model the conversion of (*E*)-8-carboxylinalool to wine lactone. Assuming that the concentration of (*E*)-8-carboxylinalool remained roughly constant over time (concentrations of wine lactone were generally *c.* 1000 times lower compared to (*E*)-8-carboxylinalool), we could estimate the first-order rate constant for wine lactone formation in wine as $k = (0.00012 \pm 0.00002)\ \mathrm{yr}^{-1}$ (Fig. 2). Because the kinetic constant was so low, we could use a linear approximation for the reaction rate, with [wine lactone] = $k \times$ [(*E*)-8-carboxylinalool] $\times$ wine age. Using this model, wine lactone concentration was underestimated in young wines with low (*E*)-8-carboxylinalool concentration. This could be due to the formation of wine lactone from (*E*)-8-carboxylinalool glucose ester in grape berries or by yeast during fermentation.

Glycosylated precursors of aroma compounds can be cleaved by enzymatic or acidic hydrolysis, both of which occur during the winemaking process (Maicas & Mateo, 2005). Accordingly, the concentration of (*E*)-8-carboxylinalool glucose ester is negatively correlated with free (*E*)-8-carboxylinalool (Fig. S1). The ratio between free and conjugated (*E*)-8-carboxylinalool is dependent on the wine variety. In Gewurztraminer, (*E*)-8-carboxylinalool glucose ester was only detected in 1-yr-old wines, whereas older wines contained only free (*E*)-8-carboxylinalool. Conversely, Riesling and Muscat wines contained (*E*)-8-carboxylinalool glucose ester even after 7 and 9 yr, respectively.

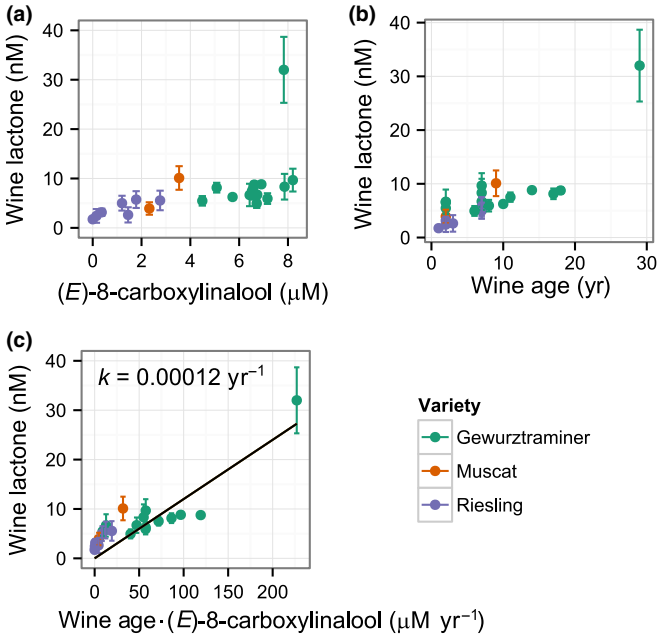

Fig. 2 Effect of wine age and precursor concentration on wine lactone content in wines. (*E*)-8-carboxylinalool, (*E*)-8-carboxylinalool glucose ester and wine lactone were quantified in the same wines by LC-MS/MS ($n = 3$, Error bars, ± SD). Concentration of wine lactone is higher in wines with a higher concentration of its precursor, (*E*)-8-carboxylinalool (a) and in older wines (b). The first-order kinetic rate constant for wine lactone formation was estimated at 0.00012 yr^{-1} (c).

Expression of candidate P450 genes parallels increasing (*E*)-8-carboxylinalool concentration during grape berry ripening

The concentrations of many grape volatiles, including oxygenated monoterpene derivatives, are higher in ripe than in green berries (Wilson *et al.*, 1984; Martin *et al.*, 2012). To establish the profile of (*E*)-8-carboxylinalool accumulation we analyzed its content in developing Gewurztraminer berries. Free (*E*)-8-carboxylinalool was not detected at any developmental stage. We could, however, detect its conjugated derivative, putatively identified as (*E*)-8-carboxylinalool glucose ester. The concentration of the conjugate increased during Gewurztraminer berry ripening (Fig. 3).

We postulated that the expression of (*E*)-8-carboxylinalool biosynthetic genes would parallel or slightly precede accumulation of (*E*)-8-carboxylinalool glucose ester in ripening grape berries. In a first step, we compared gene expression in Gewurztraminer berries at two developmental stages (green and mid-ripe) by RNA sequencing (RNA-Seq), with main focus on members of the CYP71 and CYP76 families recently shown to metabolize linalool in *A. thaliana* (Ginglinger *et al.*, 2013; Hofer *et al.*, 2014; Boachon *et al.*, 2015). The CYP76 family is larger in the *Vitis vinifera* genome than in most other plant genomes (Nelson *et al.*, 2008). Twenty full-length CYP76 coding sequences have been annotated in the reference PN40024 genome. The CYP71 family, however, is of comparable size or smaller in

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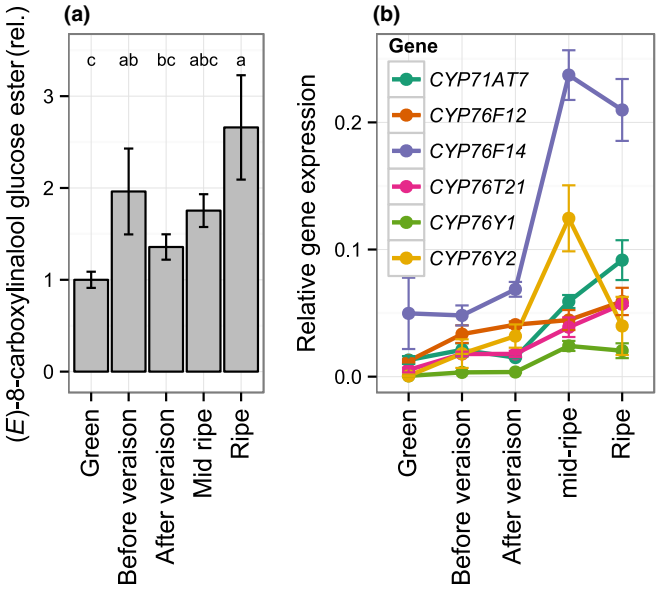

Fig. 3 Comparative increase in (*E*)-8-carboxylinalool glucose ester content and candidate P450 gene expression during grape berry development. (a) Relative concentration (± SD) of (*E*)-8-carboxylinalool glucose ester in developing Gewurztraminer berries. One-way ANOVA revealed significant differences between developmental stages ($P = 0.0016$). Letters indicate significant differences among the means calculated with Tukey–Kramer honest significant difference test ($\alpha = 0.05$). (b) Expression of candidate genes in Gewurztraminer grape berries as determined by qPCR. Error bars represent ± SD of 3 technical and 3 biological replicates. Gene expression was normalized to the expression of three constitutively expressed genes: EF1-α (XM_002284928.3), UBQ_L40 (XM_002273532.2) and MDH (m) (XM_002278676.3).

grapevine than in other plant genomes and comprises 23 predicted genes. RNA-Seq of Gewurztraminer berries highlighted five CYP76 (*CYP76F12*, *CYP76F14*, *CYP76T21*, *CYP76Y1* and *CYP76Y2*) and one CYP71 (*CYP71AT7*) gene candidates activated upon berry ripening (Fig. S2).

Expression of each candidate gene was then investigated further at five developmental stages (green, before veraison, after veraison, mid-ripe and ripe) of Gewurztraminer berries by quantitative reverse transcription polymerase chain reaction (qRT-PCR). The expression of all candidates increased along ripening (Fig. 3), paralleling the accumulation of (*E*)-8-carboxylinalool. *CYP76F14* was the most highly expressed at all stages of berry ripening.

CYP76F14 synthesizes (*E*)-8-carboxylinalool *in vitro*

In order to evaluate the catalytic activity of the candidate P450s, we cloned and expressed them in yeast together with the P450 reductase ATR1 from *A. thaliana*. The genes were amplified from Muscat Ottonel (Mo) genotype. We detected single nucleotide polymorphisms (SNPs) in all of them when compared to the coding sequences deduced from the PN40024 reference genome. The *CYP76F14* gene carried the most polymorphisms (12 SNP, six nonsynonymous). We thus also expressed and functionally evaluated this variant of CYP76F14 *in vitro*. CO-differential spectroscopy (Omura & Sato, 1964) confirmed the recombinant expression of all the candidate enzymes, except CYP76Y1 (Fig. S3). To evaluate their catalytic activity, we assayed the recombinant yeast microsomal fractions with linalool, (*E*)-8-hydroxylinalool and (*E*)-8-oxolinalool, the expected precursors of (*E*)-8-carboxylinalool.

Gas chromatography of the reaction products (Fig. S3) revealed that CYP76F12, CYP76F14 and CYP76T21 converted linalool to (*E*)-8-hydroxylinalool. (*E*)-8-oxolinalool was sometimes detected as an intermediate product, but was in most cases reduced to two side products (i.e. (*E*)-8-hydroxy-6,7-dihydrolinalool and (*E*)-8-oxo-6,7-dihydrolinalool) identified previously (Boachon *et al.*, 2015). These two products presumably were generated by an endogenous membrane-associated yeast enzyme, and also were detected in the negative control assays where (*E*)-8-oxolinalool was incubated with microsomes from yeast transformed with an empty vector.

CYP76F12 differed from the other two enzymes in that it produced both (*E*) and (*Z*) isomers of 8-hydroxylinalool. No major product could be detected for CYP71AT7, despite the observed consumption of low amounts of substrate.

The same three enzymes (CYP76F12, CYP76F14 and CYP76T21) also metabolized (*E*)-8-hydroxylinalool. Despite its low concentration in yeast microsomes (Fig. S3), CYP76F14 was the most active enzyme on both linalool and (*E*)-8-hydroxylinalool. The main products detected by GC were the two downstream products of (*E*)-8-oxolinalool, (*E*)-8-hydroxy-6,7-dihydrolinalool and (*E*)-8-oxo-6,7-dihydrolinalool.

LC-MS/MS analysis confirmed the products already identified by GC, and in addition allowed for the detection of the final product, (*E*)-8-carboxylinalool, which is not readily detectable by GC (Giaccio *et al.*, 2011). It showed that only one of the candidates, CYP76F14, catalyzed the conversion of linalool to (*E*)-8-carboxylinalool (Fig. 4). Likewise, when (*E*)-8-hydroxylinalool was used as a substrate, only CYP76F14 could convert it to (*E*)-8-carboxylinalool (Fig. S4). However, when (*E*)-8-oxolinalool was used as a substrate, the three enzymes, CYP76F14, CYP76F12 and CYP76T21, catalyzed its oxidation to to (*E*)-8-carboxylinalool (Fig. S4). CYP76F14 is thus the only candidate that catalyzes the whole oxidation cascade from linalool to (*E*)-8-carboxylinalool, mainly because of its ability to efficiently oxidize (*E*)-8-hydroxylinalool to (*E*)-8-oxolinalool. The product profile of the PN40024 CYP76F14 did not differ from that of the Muscat Ottonel enzyme *in vitro*.

Reconstruction of the (*E*)-8-carboxylinalool pathway in *Nicotiana benthamiana* leaves

In order to validate the activity of the candidate P450 enzymes *in planta*, they were transiently expressed in leaves of *N. benthamiana*. To provide them with linalool substrate, a linalool synthase (*LIS*) coding sequence (*TPS56*) was amplified from grape berry cDNA (Martin *et al.*, 2010). We transiently co-expressed *LIS* with each of the three best candidate P450 genes in leaves of *N. benthamiana* and analyzed leaf extracts using LC-MS/MS (Figs 5, S6). Although linalool, (*E*)-8-hydroxylinalool and (*E*)-8-carboxylinalool were detected in both free and conjugated forms, neither free nor glycosylated (*E*)-8-oxolinalool was

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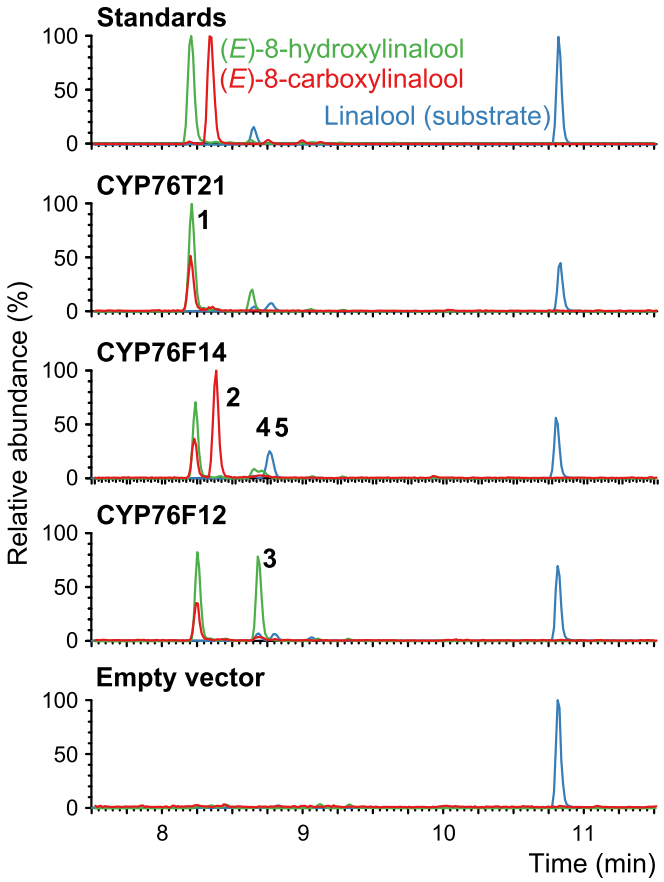

Fig. 4 Functional characterization of the candidate grapevine cytochromes P450. Microsomal membranes prepared from yeast expressing the candidate P450s were incubated with substrates at a 100 μM concentration for 1 h in the presence of NADPH and analyzed by LC-MS/MS in multiple-reaction-monitoring (MRM) mode. Each color represents an MRM channel for specific detection of target molecules: blue for linalool (137 > 80.7), green for (*E*)-8-hydroxylinalool (135 > 106.8) and red for (E)-8-carboxylinalool (167.2 > 92.8). Mass spectra of identified and putative products are available in supplementary information (Supporting Information Fig. S5). Negative control is a reaction with microsomes from yeast transformed with an empty vector. 1, (*E*)-8-hydroxylinalool; 2, (*E*)-8-carboxylinalool; 3, (*Z*)-8-hydroxylinalool; 4, (*E*)-8-oxo-6,7-dihydrolinalool; 5, (*E*)-8-hydroxy-6,7-dihydrolinalool. 4 and 5 are side products of yeast metabolism of (*E*)-8-oxolinalool.

detected in any of the samples. We also compared *in planta* activities of PN40024 and Muscat alleles of CYP76F14, but we could not detect any significant difference in product profile. Both CYP76F14 and CYP76T21 depleted all the linalool produced by LIS, but only CYP76F14 completely converted it to (*E*)-8-carboxylinalool without accumulation of the intermediate (*E*)-8-hydroxylinalool. The products of candidate enzymes in plant tissues thus confirm their *in vitro* activity, in particular the (*E*)-8-carboxylinalool synthase activity of CYP76F14.

A quantitative trait locus for (*E*)-8-carboxylinalool content in grape berries co-locates with the *CYP76F14* gene

In order to confirm the role of CYP76F14 as the main (*E*)-8-carboxylinalool synthase in grapes, analysis of QTL for (*E*)-8-carboxylinalool content in grape berries was performed on F_1 progeny from a cross between Riesling and Gewurztraminer (Ri × Gw) varieties. Three significant QTLs ($P < 0.001$ genome-wide) were detected with the interval mapping method and were included in a QTL model (Fig. S7). The first QTL explained 41.8% of the variation of the total (*E*)-8-carboxylinalool content, free and conjugated forms. It co-located with a deoxy-D-xylulose phosphate synthase gene (*DXS*), an early monoterpene biosynthesis gene (Fig. 1), which has been described previously as being involved in the accumulation of monoterpenols in grape berries (Battilana *et al.*, 2009; Duchêne *et al.*, 2009). The second QTL, explaining 12% of variation in (*E*)-8-carboxylinalool, co-located with a marker localized on the unanchored scaffold 271 in the PN40024 reference genome sequence. This scaffold contains several genes encoding terpene synthases (Martin *et al.*, 2010), consistent with a potential role in the biosynthesis of the carboxylinalool

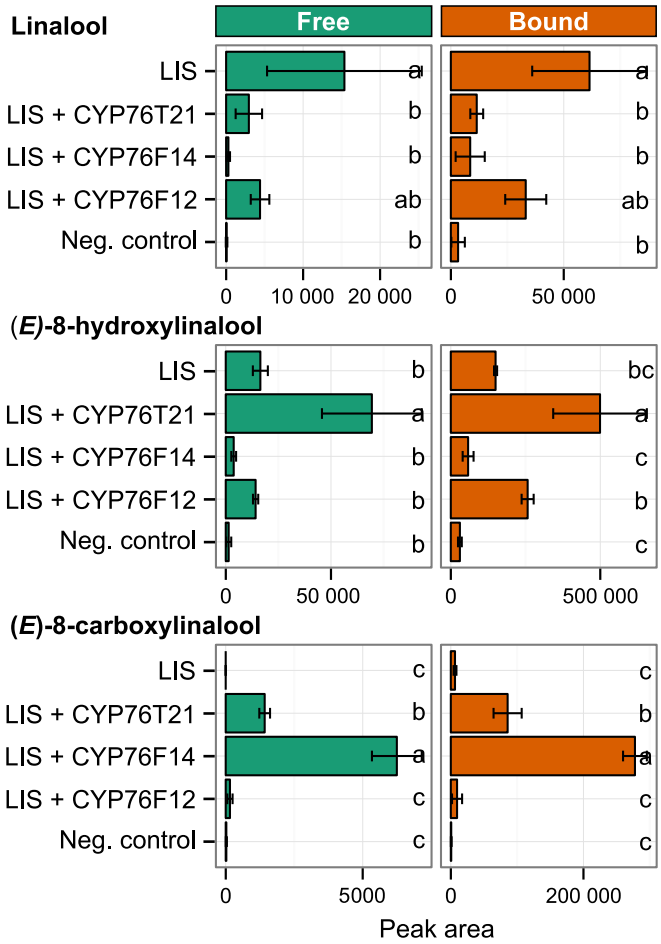

Fig. 5 Reconstitution of the (*E*)-8-carboxylinalool pathway in *Nicotiana benthamiana* leaves. *Agrobacterium*-mediated transient co-expression of linalool synthase and candidate P450 genes was performed in *N. benthamiana* leaves. Leaves expressing green fluorescent protein (GFP) were used as a negative control. Amounts of metabolites detected by LC-MS/MS are represented as means of peak areas in the corresponding MRM channel (± SD, $n = 3$). Free and bound forms of the same metabolite were detected in the same MRM channel. One-way ANOVA revealed significant differences between groups ($P < 0.05$ for linalool, $P < 0.01$ for malonylhexosyl linalool and $P < 0.001$ for other four metabolites). Letters on the right axes indicate significant differences between means calculated with the Tukey–Kramer honest significant difference test ($\alpha = 0.05$).

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precursor linalool (Fig. 1). The third QTL explained up to 7.6% of the variation and was localized on linkage group 2. Six full-length CYP genes, including *CYP76F14*, were found in the calculated confidence interval (Fig. S7). Out of them, *CYP76F14* had the highest expression level in mid-ripe berries.

Discussion

In order to gain better insight into the formation of wine lactone in wine, we measured the content of (*E*)-8-carboxylinalool, (*E*)-8-carboxylinalool glucose ester and wine lactone in 23 white wine samples.

Wine lactone was shown to form from (*E*)-8-carboxylinalool via a slow nonenzymatic cyclisation (Luan *et al.*, 2006). Wedler *et al.* (2015) recently challenged this unusual mechanism and, based on a quantum chemistry approach, suggested formation through oxidation of cyclic terpenes such as limonene or α-terpineol. We demonstrate here that the concentration of wine lactone correlates with both wine age and (*E*)-8-carboxylinalool concentration. In addition, oxygenated limonene or α-terpineol derivatives have to our knowledge never been detected in wines. Formation of wine lactone from (*E*)-8-carboxylinalool is therefore the most likely scenario in wine. It does not, however, exclude the possibility that wine lactone results from limonene oxidation in plant tissues where limonene is the main terpene constituent, such as *Citrus* fruits.

The flavor of wines is known to change with aging, yet the underlying chemical changes are poorly understood. We found that the formation of wine lactone from (*E*)-8-carboxylinalool in wine follows first-order reaction kinetics with an extremely low reaction rate constant, $k = 0.00012\ \mathrm{yr}^{-1}$. This slow reaction rate is in agreement with the high activation energy required for the unlikely hydride shift proposed in the reaction mechanism (Luan *et al.*, 2006). The resulting low amounts of wine lactone are nonetheless sufficient to affect wine aroma due to the extremely potent odor of this compound.

All (*E*)-8-carboxylinalool is stored in grape berries as a glucose ester. Conversely, it is present in both the free and conjugated forms in young wines, suggesting that the aglycone is partially released during fermentation (Giaccio *et al.*, 2015). In older wines, the glucose ester further decreases, with a concomitant increase in free (*E*)-8-carboxylinalool.

We found large differences in wine lactone contents between different wines, stemming from differences in wine age and (*E*)-8-carboxylinalool content. Of the three wine varieties analyzed, the highest concentrations were measured in Gewurztraminer wines, supporting the role of wine lactone as prime Gewurztraminer varietal aroma compound (Guth, 1997b). Our data show this is likely due to high concentration of the wine lactone precursor (*E*)-8-carboxylinalool in Gewurztraminer wines.

We identified six candidate P450 enzymes potentially involved in (*E*)-8-carboxylinalool biosynthesis in grape, based on their increased expression in ripe berries, which parallels (*E*)-8-carboxylinalool accumulation. Although three of them could metabolize linalool, both *in vitro* activities and the pathway reconstruction in *N. benthamiana* demonstrate that only CYP76F14 catalyzes the whole oxidation cascade to (*E*)-8-carboxylinalool. In addition, of all candidates, *CYP76F14* has the highest expression in maturing berries. A terpene synthase (LIS) and CYP76F14 are sufficient to produce (*E*)-8-carboxylinalool from common plant precursors in *N. benthamiana*.

Two (*E*)-8-carboxylinalool biosynthesis intermediates, linalool and (*E*)-8-hydroxylinalool, accumulate in grapes as glycosylated derivatives (Strauss *et al.*, 1988), the formation of which requires UDP glycosyltransferase (UGT) activity. Because UGTs compete with P450 enzymes for available linalool and (*E*)-8-hydroxylinalool, they can prevent further enzymatic oxidation and formation of the final product. In reconstruction assays, *N. benthamiana* leaves expressing CYP76F14 accumulated only (*E*)-8-carboxylinalool, but no linalool nor (*E*)-8-hydroxylinalool, either in free or glycosylated form. Thus, CYP76F14 can efficiently outcompete UGTs, in stark contrast to the other P450 candidates. *N. benthamiana* leaves co-expressing LIS and either CYP76T21 or CYP76F12 accumulated significant amounts of glycosylated linalool and (*E*)-8-hydroxylinalool, and minor amounts of (*E*)-8-carboxylinalool compared to CYP76F14.

In order to investigate the genetic basis of carboxylinalool biosynthesis in grapevine, we analyzed the berries of F_1 Ri × Gw progeny for their (*E*)-8-carboxylinalool content. In this progeny, carboxylinalool accumulation depended on three major QTLs, one of them co-localizing with CYP76F14 and two co-localizing with linalool biosynthesis genes upstream of CYP76F14. In addition to the transcriptomic and functional data, these genetic data strongly support the role of CYP76F14 as the main (*E*)-8-carboxylinalool synthase in grapes. After monoterpenol biosynthesis gene *DXS* (Battilana *et al.*, 2009; Duchêne *et al.*, 2009) and methoxpyrazine producing *O*-methyl transferase gene *OMT3* (Dunlevy *et al.*, 2013; Guillaumie *et al.*, 2013), this is only the third wine aroma gene, whose role *in planta* is confirmed by genetic data. These results also point to the complexity of aroma traits in grapevine, with (*E*)-8-carboxylinalool content controlled by three loci at three different points of the biosynthetic pathway (Fig. 1). In addition to the biosynthesis of the wine lactone precursor, CYP76F14 may also influence wine aroma by depleting linalool and other monoterpenols. CYP76F14 could thus be used as a marker for the selection of grapevine varieties producing desired amounts of monoterpenols and wine lactone precursor.

The wine lactone molecule has three chiral centers, resulting in eight possible stereoisomers. Only one of them, the (3*S*,3a*S*,7a*R*) form, is a powerful odorant and until recently was the only stereoisomer detected in wines (Guth, 1996). Interestingly, it results from the cyclisation of (3*R*, 6*E*)-8-carboxylinalool, whereas the main linalool enantiomer in wine is (3*S*)-linalool (Luan *et al.*, 2004). Recent work confirmed that the predominant configuration of (*E*)-8-carboxylinalool in wine is also (3*S*), although the ratio between enantiomers varied between samples (Giaccio *et al.*, 2015). Consistent with the presence of both (*E*)-8-carboxylinalool enantiomers, the same study also found two enantiomers of wine lactone in wine, the more abundant one being the less fragrant (3*R*,3a*R*,7a*S*) enantiomer. We tested CYP76F14 with both (*R*)- and (*S*)-linalool and it did not distinguish between these two forms (Fig. S3). The enzyme is therefore not expected to influence the wine lactone enantiomeric ratio.

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We showed that (*E*)-8-carboxylinalool accumulates in grape berries as glucose ester. (*E*)-8-carboxylinalool was not included as a substrate in the recently reported characterization of grapevine UGTs (Bönisch *et al.*, 2014a,b), so the enzyme catalyzing this reaction remains to be discovered. Profiling of wines showed that hydrolysis of this glucose ester occurs both during fermentation and during wine maturation. The role of yeast in this process remains to be explored and could be an important factor affecting the wine lactone formation.

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Author contributions

T.I., N.N., E.D., P.H. and D.W-R. designed the study; T.I., D.H, L.K., A.I., R.B. and E.D. performed all experiments; F.L. and L.M. performed chemical synthesis; T.I., N.N., E.D., P.H. and D.W-R. analyzed the data and wrote the manuscript.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information tab for this article:

Fig. S1 Relationship between (*E*)-8-carboxylinalool glucose ester and wine age, and free (*E*)-8-carboxylinalool in 23 wine samples.

Fig. S2 Expression of *CYP71* and *CYP76* genes at two different developmental stages of Gewurztraminer berries.

Fig. S3 GC-FID characterization of *in vitro* linalool and (*E*)-8-hydroxylinalool metabolism by the candidate grapevine cytochromes P450 expressed in yeast.

Fig. S4 LC-MS/MS analysis of the products of (*E*)-8-hydroxylinalool and (*E*)-8-oxolinalool metabolism by the yeast-expressed candidate grapevine cytochromes P450.

Fig. S5 Mass spectra (ESI-MS) of the products identified by comparison with authentic standards or putatively identified products.

Fig. S6 LC-MS/MS targeted analysis of the *N. benthamiana* leaves expressing the grapevine LIS and each of the P450 candidates.

Fig. S7 Genetic mapping of the (*E*)-8-carboxylinalool–associated QTLs in grape berries.

Table S1 Putative peak annotation in *N. benthamiana* extracts

Table S2 Primers used for gene expression quantification in a qRT-PCR experiment

Table S3 Primers used for amplification of coding sequences

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