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BIOTECHNOLOGICAL PRODUCTS AND PROCESS ENGINEERING

Novel wine yeast with *ARO4* and *TYR1* mutations that overproduce 'floral' aroma compounds 2-phenylethanol and 2-phenylethyl acetate

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Abstract

It is well established that the choice of yeast used to perform wine fermentation significantly influences the sensory attributes of wines; different yeast species and strains impart different profiles of esters, volatile fatty acids, higher alcohols, and volatile sulphur compounds. Indeed, choice of yeast remains one of the simplest means by which winemakers can modulate the sensory characteristics of wine. Consequently, there are more than 100 commercially available *Saccharomyces cerevisiae* wine yeast strains available, mostly derived by isolation from vineyards and successful fermentations. Nevertheless, some desirable characteristics such as 'rose' and 'floral' aromas in wine are not present amongst existing strains. Such aromas can be conferred from the higher alcohol 2-phenylethanol (2-PE) and its acetate ester, 2-phenylethyl acetate (2-PEA). These metabolites of the aromatic amino acid phenylalanine are present at concentrations below their aroma detection thresholds in many wines, so their contribution to wine style is often minimal. To increase the concentration of phenylalanine metabolites, natural and chemically mutagenised populations of a *S. cerevisiae* wine strain, AWRI796, were exposed to toxic analogues of phenylalanine. Resistant colonies were found to overproduce 2-PE and 2-PEA by up to 20-fold, which resulted in a significant increase in 'floral' aroma in pilot-scale white wines. Genome sequencing of these newly developed strains revealed mutations in two genes of the biosynthetic pathway of aromatic amino acids, *ARO4* and *TYR1*, which were demonstrated to be responsible for the 2-PE overproduction phenotype.

Keywords Amino acid · Yeast · Wine · 2-Phenylethanol · Aroma

Introduction

Floral aromas are prominent in a small number of aromatic white grape cultivars such as Muscat type grapes and Gewürztraminer (Marais 1983), but are also noted amongst descriptors for many other varieties (Fang and Qian 2005; Gürbüz et al. 2006). These aromas are predominantly associated with grape-derived monoterpenes such as linalool and geraniol, found at relatively high concentrations in these aromatic varietals (Marais 1983; Vilanova et al. 2013). Recent studies have characterised *Vitis vinifera* monoterpene synthases with the goal of enhancing monoterpene content in non-aromatic varieties (Martin et al. 2010), and yeast have been genetically modified (GM) to produce significant quantities of monoterpenes such as geraniol (Fischer et al. 2011). Non-GM options to enhance floral aromas by increasing monoterpene content of wine made from existing grape varieties remain limited, though there is some potential to harness β-glucosidase activity of some wine bacteria (Michlmayr et al. 2012) and yeast (Ugliano et al. 2006) to release glycosidically bound monoterpenes.

Another source of floral aromas are the higher alcohol 2-phenylethanol (2-PE) and its acetate ester 2-phenylethyl acetate (2-PEA), which impart 'rose'-like and 'fruity', 'honey',

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and 'rose'-like aromas, respectively (Swiegers et al. 2005). These predominantly yeast-derived compounds are typically found at concentrations below their aroma detection thresholds in white varietals (Rodríguez-Bencomo et al. 2002; Vilanova et al. 2013), though their production can be enhanced by *Saccharomyces* interspecies hybrids (Bellon et al. 2013).

Higher alcohols, also known as fusel alcohols, are derived from yeast metabolism by one of the two following pathways: catabolism of grape amino acids via the Ehrlich pathway, or production of α-keto acids during amino acid biosynthesis from sugars, also known as the anabolic pathway (Hazelwood et al. 2008; Nisbet et al. 2014). With the exception of 2-PE, their aroma contribution to wine is not considered to be pleasant, particularly at high concentrations (de la Fuente Blanco et al. 2016; Etiévant 1991). Higher alcohols are important precursors for ester production, and esters of higher alcohols are generally associated with desirable aromas: for example, both 2-PE (rose-like) and 3-methylbutanol (harsh, nail polish) can be esterified and converted into 2-PEA (rose, fruity) and 3-methylbutyl acetate (banana), respectively.

Viticultural conditions and the choice of yeast strain during alcoholic fermentation contribute considerably to variations in the profile and concentrations of higher alcohols (Giudici et al. 1990). The concentration of amino acids in the must is also an important factor influencing the production of higher alcohols: increased concentrations of a specific precursor amino acid will result in greater formation of the corresponding higher alcohol through the Ehrlich pathway (Bordiga et al. 2016; Hernández-Orte et al. 2002; Schulthess and Ettlinger 1978). Therefore, one strategy to increase the formation of higher alcohols is to select for a yeast strain that overproduces the corresponding precursor. For 2-PE, the target for overproduction is the aromatic amino acid L-phenylalanine (L-Phe) (Fukuda et al. 1990a; Fukuda et al. 1990b; Dueñas-Sánchez et al. 2014).

The biosynthesis of aromatic amino acids in *S. cerevisiae* is controlled at multiple levels, including end-product feedback inhibition at different checkpoints of the pathway. Extensive research has been carried out to find ways to increase the production of these compounds by removing these feedback inhibition points since the aromatic biosynthetic pathway is a source for precursors to many commercially relevant chemicals such as opioids and vanillin (reviewed by Suástegui and Shao 2016).

One non-GM strategy successfully applied for industrial yeast strain improvement involves the use of toxic amino acid analogues that select for cells with decreased feedback inhibition of amino acid synthesis, thereby causing amino acid overproduction and metabolic overflow to corresponding higher alcohols. In particular, increased production of the higher alcohols 3-methylbutanol, tyrosol, and 2-PE has been achieved for sake and baker's yeast by selecting mutants with resistance to toxic fluorinated amino acid analogues of L-leucine (L-Leu) (Oba et al. 2005), L-tyrosine (L-Tyr) (Koseki et al. 2004), and L-Phe, respectively (Fukuda et al. 1990a; Fukuda et al. 1990b; Dueñas-Sánchez et al. 2014).

In this study, we isolated variants of the wine yeast strain AWRI796 that were resistant to toxic analogues of the aromatic amino acid L-Phe, *p*-fluoro-DL-phenylalanine (PFP), and *o*-fluoro-DL-phenylalanine (OFP). We show that these mutants can be utilised to produce substantial quantities of 2-PE and 2-PEA during winemaking, with a high degree of specificity, significantly altering the sensory profile of resultant wine. Finally, we demonstrate that mutations in *ARO4* and *TYR1* were induced by OFP and PFP, respectively, and these mutations are responsible for the observed 'floral' aroma phenotype.

Materials and methods

Microorganisms and culture conditions

The diploid wine strain AWRI796 was obtained from The Australian Wine Research Institute (AWRI) culture collection, and mutants generated in this work were deposited into the collection. AWRI796 was chosen as a suitable parent strain on the basis of its moderately heterozygous genome (Borneman et al. 2016) and relatively low production of aroma compounds. Yeast cultures were maintained on solid YPD agar plates (2% glucose, 2% peptone, 1% yeast extract, and 2% agar). Minimal synthetic medium (MSM) plates (0.67% yeast nitrogen base without amino acids (Difco), and 2% glucose) supplemented with either *p*-fluoro-DL-phenylalanine (PFP, Sigma F5251) or *o*-fluoro-DL-phenylalanine (OFP, 47300 Aldrich) were used to isolate PFP- and OFP-resistant strains, respectively. Yeast strains were transformed using the lithium acetate procedure (Schiestl and Gietz 1989). Grape juice used for fermentation trials was cold-settled at 4 °C for 48 h and filtered through 0.22-μm Stericup filters (Millipore).

Mutagenesis and strain isolation

Mutants were generated using a classical mutagenesis technique (Lawrence 1991). A concentration of 6% of the alkylating agent ethyl methane sulfonate (EMS, Sigma M0880) was used to mutagenise AWRI796, as described in Cordente et al. (2009). After stopping mutagenesis with sodium thiosulfate, cells were then washed twice with sterile water, grown for 3 h in liquid YPD, and 2×10^8 cells were spread on either MSM-PFP 2 mg mL^{-1} or MSM-OFP 1 mg mL^{-1}. Mutants appearing after 3–4 days of incubation at 30 °C were re-streaked three times to isolate stable-resistant colonies. The parental AWRI796 strain was able to grow up to a concentration of 50 mg L^{-1} PFP and 20 mg L^{-1} OFP.

Screening of resistant strains

The isolated PFP- and OFP-resistant strains were screened for 'floral' aroma production by performing 30 mL un-replicated ferments of diluted Chardonnay juice (50% in Milli-Q H_2O), in 50-mL plastic centrifuge tubes that were modified to enable CO_2 release by insertion of 0.2-mL pipette tips through tube caps. After 2 weeks of fermentation at 17 °C, an informal sensory assessment of the wines was performed, whereby each sample was scored for 'floral'/'rose' aromas.

Laboratory-scale fermentation in Chardonnay juice

Fermentations were performed in duplicate in 250-mL conical flasks fitted with an air-lock and side-arm port sealed with a rubber septum for sampling. Fermentations were incubated at 17 °C with shaking (100 rpm), and their progress was monitored by CO_2 loss. Yeast starter cultures were prepared in YPD medium, incubated aerobically at 28 °C with shaking for 24 h to stationary phase. Then, a concentration of 1×10^6 cells mL^{-1} was inoculated in a 50% dilution of the same Chardonnay juice for 24 h, and the acclimatised cells were inoculated into 200 mL of a Chardonnay grape juice at a density of 2×10^6 cells mL^{-1}. Basic chemical parameters of the Chardonnay juice were pH 3.41, sugars 232 g L^{-1}, yeast assimilable nitrogen 227 mg L^{-1}, and free and total SO_2 17 and 50 mg L^{-1}, respectively. Fermentation progress was monitored by measuring weight loss, and fermentations were considered complete when weights were static for 2 days. At the end of the fermentation, samples were centrifuged for 5 min at 5000×*g* and the cell-free supernatants were stored at − 20 °C for analysis.

Pilot-scale winemaking

Pilot-scale winemaking trial was performed using a Chardonnay juice from the Adelaide Hills (South Australia, Australia) by Wine Innovation Cluster (WIC) winemaking services according to a standardised winemaking protocol. Nineteen litters of ferments was conducted in triplicate at 15 °C for both the parent AWRI796 strain and one of the 2-PE overproducing spontaneous mutants, AWRI2940. The basic chemical parameters of the juice were as follows: Baumé 12.7, yeast assimilable nitrogen 119 mg L^{-1} and pH 3.32. Once the alcoholic fermentation had finished, the wines were sulphured, cold-stabilised and bottled for sensory and chemical analysis according to standard WIC winemaking protocols.

Minimum inhibitory concentration of toxic analogues of phenylalanine

Strains were cultured in liquid MSM-2% glucose medium overnight at 28 °C. Then, 5 μL of the culture was spotted in MSM agar plates in serial dilutions (1/10–1/100,000) containing 10–500 mg L^{-1} PFP, and growth of the cells was monitored after incubation for 4 days at 30 °C.

Analysis of principal non-volatile compounds

The concentrations of sugars, ethanol, glycerol, and organic acids were measured by high-performance liquid chromatography (HPLC) using a Bio-Rad HPX-87H column, as described previously (Nissen et al. 1997). Total phenolics in the finished wines were analysed according to the method of Somers and Evans (1974).

Intracellular metabolite extraction was performed using the method described by Spura et al. (2009). Quantitation of amino acids was performed by Metabolomics Australia (Adelaide), using a derivatization technique with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate, as described in Boughton et al. (2011). Amino acids were analysed by LC-MS/MS using an Agilent 1290SL HPLC system coupled to an Agilent 6490A QqQ-MS, and quantitated using the Agilent MassHunter software.

Tyrosol and tryptophol were analysed on an Agilent 1200SL HPLC using a Phenomenex Kinetex® PFP column (2.6-μm particle size, 2.1 × 150 mm). The injection volume was 5 μL. The column was eluted at 45 °C with a gradient of 0.1% formic acid in Milli-Q water (A) and 0.1% formic acid in acetonitrile (B), at a flow rate of 0.4 mL min^{-1}. The gradient was as follows: an initial isocratic hold (0% B) for 8 min, then ramp gradient to 5% over the next 32 min, ramp gradient to 25% over 9 min, then gradient to 80% over 3 min, held isocratically at 80% for 3 min, and dropped to 0% and held for another 15 min. Absorbance at 280 nm was monitored throughout the process with an Agilent 1200 Series G1315D DAD, and tyrosol and tryptophol were detected at 9 and 39 min, respectively.

Targeted analyses of volatile compounds

Targeted analyses of fermentation-derived higher alcohols, acids, and esters were performed by Metabolomics Australia (Adelaide) on wine samples by GC-MS using a stable isotope dilution assay as previously described (Siebert et al. 2005) with some modifications (Bizaj et al. 2012).

Sensory evaluation

A sensory descriptive analysis was carried out using a panel of ten trained and experienced members of the AWRI sensory panel (three males and seven females). A preliminary attribute list containing descriptors for appearance, aroma and palate was drafted from the tasting notes from an informal assessment and from attributes rated in similar sensory studies. Following a single training discussion session to refine the

attribute list, tasters participated in a practice session under the same conditions as those for the formal sessions. After the practice session, any terms which needed adjustment were discussed and the final list of terms and their definitions were determined. Reference standards for the aroma attributes were developed. Samples were presented to panellists in 30 mL aliquots in 3-digit-coded, covered, ISO standard wine glasses at 22–24 °C, in isolated booths under daylight-type lighting, with randomised presentation order. Each of the wines made in triplicate from the two strains was presented to assessors twice in a modified Williams Latin Square incomplete random block design generated by Fizz sensory acquisition software (version 2.47B, Biosystemes, Couternon, France). Three wines were presented per session, with a 30-s forced rest between wines and a minimum of 10-min rest between sessions. The intensity of each attribute was rated using an unstructured 15-cm line scale from 0 to 10, with indented anchor points of 'low' and 'high' placed at 10 and 90% respectively. Data was acquired using Fizz sensory software. Panel performance was assessed using Fizz and PanelCheck (panelcheck.com) software, and included analysis of variance for the effect of judge and presentation replicate and their interactions, degree of agreement with the panel mean, and degree of discrimination across samples. Analysis of variance (ANOVA) was carried out using Minitab (Minitab Inc., Sydney, NSW) for the effects of strain, fermentation replicate nested within strain, judge, strain by judge, presentation replicate nested in ferment replicate and strain, and judge × presentation replicate nested within strain, treating judge as a random effect.

DNA isolation, extraction, and sequencing

Strains were grown overnight at 28 °C in YPD, and 2 mL of the culture was used for DNA extraction (Gentra Puregene Yeast/Bact kit, Qiagen). Nextera XT (Illumina) genomic libraries were prepared and sequenced using Illumina Miseq, paired-end 300-bp chemistry (Ramaciotti Centre for Functional Genomics, University of NSW, Australia).

Sequence processing, reference-based alignment, and variant analysis

Raw sequences were quality trimmed and aligned to a *Saccharomyces cerevisiae* reference genome as described in Borneman et al. (2016). Potential mutations were identified using the 'somatic' function of Varscan (v2.3.8 DOI: https://doi.org/10.1101/gr.129684.111), by separately comparing each mutant isolate with the parental strain (AWRI796). Candidate mutations were sorted according to significance and those with *P* values lower than 0.05 were manually curated by visualising read alignments in the Integrated Genomics Viewer (IGV) (https://doi.org/10.1093/bib/bbs017).

Chromosomal integration of mutant alleles in AWRI1631

Each of the AWRI796 *ARO4* and *TYR1* mutant alleles was inserted at their chromosomal location in the haploid wine yeast strain AWRI1631 (Borneman et al. 2008), via homologous recombination (Ausubel et al. 1994) to replace the corresponding AWRI1631 wild-type locus. Genomic DNA from strains AWRI2964, 2965, and 2967 was used as template to amplify by PCR each of the *ARO4* mutated alleles (*ARO4* G64T, A497G, and C584Y, respectively) using Phusion® High-Fidelity DNA polymerase (New England Biolabs). Each purified PCR product (1.4 kb), encompassing the full-length ORF of *ARO4* plus 125 bp before the start codon and 150 bp after the stop codon, was then transformed in the AWRI1631 *Aro4Δ* strain (obtained from the AWRI1631 deletion collection). Positive transformants were selected in MSM-PFP 50 mg L^{-1} plates, and integration of the mutate allele was confirmed by PCR and Sanger sequencing. AWRI1631 and AWRI1631 *Aro4Δ* grew up to a concentration of 20 mg L^{-1} PFP. The 'wild-type' alleles of *ARO4* in the AWRI1631 and AWRI796 backgrounds are identical.

For the integration of three of the *TYR1* mutations (*TYR1* C629T, G550A, G591T) in AWRI1631, a modification of the CRISPR-Cas9 protocol described by Ryan et al. (2016) was used. A 20-mer DNA target sequence (5′-AAGA TTTATCCTTGGACTCT-3′) was designed using the Yeastriction tool (http://yeastriction.tnw.tudelft.nl) (Mans et al. 2015), immediately 5′ of an NGG protospacer adjacent motif (PAM) sequence in AWRI1631 *TYR1* (nucleotides 633–635). This target sequence was selected on the basis of its close proximity to the mutations to be introduced, and optimal score taking into account its AT content and RNA score. The primers CRISPR-Tyr1-635.f (5′-CGGGTGGCGAATGG GACTTTAAGATTTATCCTTGGACTCTGTTT TAGAGCTAGAAATAGC-3′), and its reverse complement CRISPR-Tyr1-635.r, were designed to clone the 20-mer DNA target sequence (underlined) into the sgRNA cassette of the pCAS plasmid (Ryan et al. 2016), using a modification of the Quikchange II site-directed mutagenesis method (Agilent Technologies). Briefly, a PCR mix (50 μL) was prepared using 4 U of *Pfu*Turbo DNA polymerase (Agilent), 40 ng of pCAS plasmid, and 125 ng of each of the primers; then, the DNA was amplified at 56 °C for 18 min (2 min per kb) with 22 cycles of amplification. After the PCR reaction, the parental methylated DNA was degraded with *Dpn*I, and the PCR-generated DNA was transformed into *E. coli* competent cells and selected in kanamycin plates. The cloning of the 20-mer target sequence was confirmed by Sanger sequencing, and the resulting plasmid was named pCAS-Tyr1.

The double-stranded linear repair fragments (400 bp) containing the mutations to be integrated in the *TYR1* locus of AWRI1631 were obtained using the GeneArt Gene

Synthesis service (ThermoFisher Scientific) and amplified by PCR to yield a fragment of approximately 350 bp. In the case of the *TYR1* G550A and G591T alleles, since both mutations are further than 20 nucleotides of the PAM sequence, the repair fragments were designed so they contained not only the mutation to be integrated but an additional synonymous codon substitution (underlined) within 20 nucleotides of the PAM (5′-AAGATTTATCCCTGGACTCT-3′). Then, 100 ng of the pCAS-Tyr1 plasmid and 5 μg of the PCR-amplified repair fragment (approximately 1:1000 M ratio) were transformed into AWRI1631 using the standard lithium acetate method. Cells were selected with G418, replica-plated in MSM-PFP 100 mg L^{-1}, and then sequenced to confirm the presence of the mutated allele.

Performance of the modified AWRI1631 strains with the AWRI796 *TYR1* and *ARO4* mutated alleles was evaluated by fermentation of 200 mL synthetic grape juice (SGJ) medium (Cordente et al. 2015) with the following variations: sugar content was 100 g L^{-1} glucose and 100 g L^{-1} fructose; 250 mg L^{-1} yeast assimilable nitrogen.

Results

Random mutagenesis and screening for 'floral' phenotype

To isolate high 2-PE-producing yeast derived from the diploid commercial wine yeast AWRI796, a classical mutagenesis technique was used, based on the incubation of yeast cells with the alkylating agent EMS. A concentration of 6% EMS, which produced a survival rate of 62%, was used. A total of 62 and 58 resistant colonies were isolated in agar plates containing PFP or OFP as the toxic phenylalanine analogue, respectively. In parallel, we were also able to isolate a total of 38 spontaneous mutants from a population of cells (1×10^9 cells) that did not undergo chemical mutagenesis. As expected, the isolation rates were lower for the spontaneous versus the mutagenised populations. Isolation rates were found to be 1×10^{-6} for the mutagenised population selected in PFP plates and 3.7×10^{-8} for the spontaneous PFP-resistant colonies, whereas these figures were 8×10^{-7} and 4.5×10^{-8} for the mutagenised and spontaneous OFP-resistant colonies, respectively. All the isolated colonies grew in minimal media in which the only nitrogen source was L-Phe (20 mM), confirming that observed resistance towards toxic analogues of L-Phe was not due to a defect in the transport of this amino acid into the cell.

All 158 isolated colonies were used to perform microfermentations of a diluted Chardonnay juice (50%), and after 2 weeks of fermentation at 17 °C, an informal sensory evaluation of the resulting wines was performed. A total of 31 strains displaying a strong 'floral', 'rose'-like aroma were selected, and of these, 13 were spontaneous mutants. Chemical analysis (data not shown) confirmed that 25 of the strains selected overproduced both 2-PE and 2-PEA by at least twofold relative to the parent strain AWRI796.

Laboratory-scale validation of selected mutants

Performance of the 25 selected strains was assessed by laboratory-scale fermentation of Chardonnay juice, and all but two of the selected strains fermented at a similar rate as the AWRI796 parent (Fig. S1). Wines were analysed at the end of the fermentation, and all selected strains overproduced both 2-PE and its acetate ester 2-PEA when compared with the parent strain AWRI796 (Table 1). Regardless of the selection method used, all strains showed a similar increase in the levels of 2-PE when compared to the control AWRI796, about six-fold on average. On the other hand, strains isolated using PFP as the toxic analogue showed a much larger increase in the levels of the acetate ester 2-PEA than strains isolated using OFP. Furthermore, in strains selected using the toxic analogue PFP, no differences in the production of both 2-PE and 2-PEA were observed between the spontaneous and mutagenised groups. In the case of strains selected using OFP, both spontaneous and mutagenised strains produced similar levels of 2-PE, but the mutagenised population produced higher levels of 2-PEA than their spontaneous counterparts. A range of related products derived from the Ehrlich pathway were also found at elevated levels, though the increases were modest by comparison to 2-PE/2-PEA (Fig. 1). Notably, concentrations of the acetate esters 2-methylpropyl acetate and 2- and 3-methylbutyl acetate, derived from valine, isoleucine, and leucine, respectively, were found to be increased by the majority of resistant strains assessed in this study, and this effect was found to be more pronounced in strains selected using PFP-resistant than in the OFP-resistant strains.

Pilot-scale winemaking and sensory assessment of wines

One of the spontaneous strains selected using PFP as the toxic L-Phe analogue, AWRI2940, was chosen for pilot-scale Chardonnay juice fermentation. AWRI2940 displayed similar fermentation kinetics to the parent strain AWRI796 in laboratory-scale fermentations and was amongst the highest 2-PE and 2-PEA producers (6.6- and 14.4-fold increase, respectively).

Both the parental strain AWRI796 and the variant AWRI2940 fermented at the same rate (Fig. S2), and wines were dry (< 2 g L^{-1} residual sugar) after 24 days of fermentation at 15 °C. Implantation of the variant strain was evaluated at different stages during fermentation (25, 50, 75, and 90% of sugar fermented) by plating ferment samples into YPD plates and then replica plating into solid media containing

Table 1 Production of 2-phenylethanol (2-PE) and 2-phenylethyl acetate (2-PEA) after fermentation of 200 mL of a Chardonnay juice by strains selected using the toxic analogues *p*-fluoro-DL-phenylalanine (PFP) or *o*-fluoro-DL-phenylalanine (OFP)

Selection	*n*	2-PEA production*	2-PE production*	Ratio 2-PEA/2-PE
PFP (spontaneous mutants)	7	13.3 (9.7–16.3)[a]	6 (4.7–6.6)[a]	2.2[a]
PFP (EMS mutagenesis)	10	12.7 (6–17.7)[a]	5.9 (3.5–7.2)[a]	2.1[a]
OFP (spontaneous mutants)	4	3.0 (1.7–4.2)[b]	5.6 (2.6–9.9)[a]	0.55[b]
OFP (EMS mutagenesis)	4	5.3 (2.1–9.1)[b]	6.2 (3.6–8.5)[a]	0.79[b]

n number of strains in each of the selection methods

*Results are expressed as the average of the fold change relative to the parent strain AWRI796. The range, in the formation of 2-PE and 2-PEA, in strains obtained using the same selection method is indicated in parenthesis. Means with the same superscript letter are not significantly different from each other (Tukey's test, $P < 0.05$)

1 mg mL^{-1} of PFP. Between 93 and 96% of the yeast population was PFP-resistant, confirming that AWRI2940 was responsible for fermentation. Growth on PFP plates was not observed for samples taken from ferments inoculated with the parental strain AWRI796.

Chemical analysis of the wines 6 months after bottling confirmed the 2-PE/2-PEA overproduction phenotype of strain AWRI2940 (Fig. 2). There was a very large, 37-fold, increase in the concentration of 2-PEA in wines made with strain AWRI2940 when compared to the control strain AWRI796. Importantly, while the concentration of 2-PEA was well below its odour detection threshold in wines made with the control strain AWRI796, concentrations almost five times this threshold were observed for wines made with AWRI2940. In addition, we observed a sevenfold increase in the concentrations of 2-PE in wines made with strain AWRI2940 with respect to the control strain. In this case, 2-PE was well above the odour threshold for all wines.

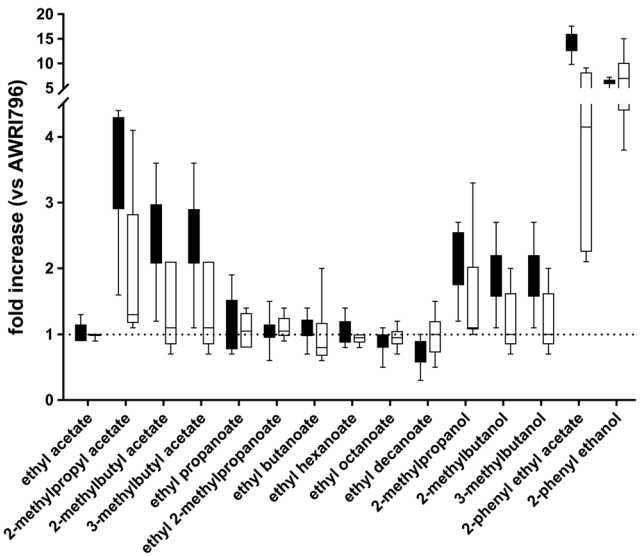

Fig. 1 Production of esters and fusel alcohols, after fermentation of 200 mL of a Chardonnay juice, by strains selected using the toxic analogues of phenylalanine PFP (black) or OFP (white). Results are expressed as the average of the fold change in the concentration of these fermentation products relative to the parent strain AWRI796 (control). A total of 17 PFP- and 8 OFP-resistant strains are shown in the figure

As seen at laboratory-scale, pilot-scale wines made with AWRI2940 were also characterised by elevated concentrations of other higher alcohols and their corresponding acetate esters, all of which are formed via the Ehrlich pathway (Table 2). The levels of both 2- and 3-methylbutanol and their corresponding acetate esters 2- and 3-methylbutyl acetate were significantly higher in wines made with the strain AWRI2940. A small, but significant increase in some medium chain ethyl esters was also observed. While most non-volatile wine components were unaffected by fermentation with AWRI2940 relative to AWRI796, total phenolic content was significantly higher ($P < 0.05$). Phenolic higher alcohols tryptophol and tyrosol, respectively derived from tryptophol and tyrosine, were analysed in the pilot-scale wines (Table 2).

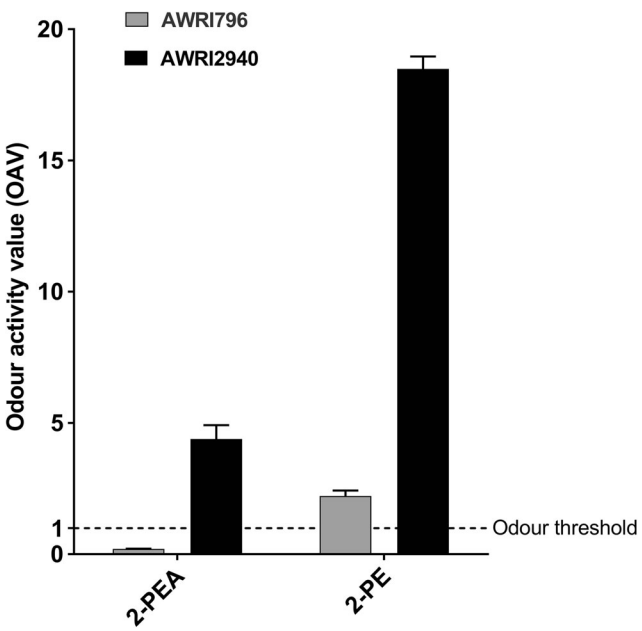

Fig. 2 Odour activity value (OAV) for 2-phenylethanol (2-PE) and 2-phenylethyl acetate (2-PEA) in the pilot-scale Chardonnay wines (19 L) produced with the parent strain AWRI796 (grey) and the spontaneous PFP-resistant strain AWRI2940 (black). Data is expressed as the mean and standard deviation of three fermentation replicates. An OAV higher than 1 indicates a possible contribution of the compound to the final aroma of wine. The odour threshold was 0.25 mg L^{-1} for 2-PEA and 10 mg L^{-1} for 2-PE (Guth 1997; Swiegers et al. 2005)

Table 2 Main fermentation products (g L^{-1}) and volatile compounds (μg L^{-1}) produced at the end of fermentation of 19 L of a Chardonnay grape juice with the parent strain AWRI796 and the spontaneous PFP-resistant strain AWRI2940

Metabolite	AWRI796	AWRI2940
Main fermentation products (g L^{-1})		
Tartaric acid	2.9	2.7
Malic acid	2.1	2.3
Succinic acid	1.9	1.9
Lactic acid	0.32	0.41
Glycerol	10.5	10.8
Volatile acidity	0.42	0.43
Residual sugar	0.8	1.2
Ethanol (%, *v/v*)	13.6	13.5
Volatile compounds (μg L^{-1})		
Ethyl acetate	41,248	41,621
Ethyl propanoate	400	389
Ethyl 2-methylpropanoate	63	85
2-Methylpropyl acetate (Val)	21	31*
2-Phenylethyl acetate (Phe)	51	1098***
2-Methylbutyl acetate (Ile)	98	165***
3-Methylbutyl acetate (Leu)	673	1334***
Ethyl butanoate	300	467**
Ethyl hexanoate	70	96**
Ethyl octanoate	95	113*
Ethyl decanoate	23	24
2-Methylpropanol (Val)	58,280	61,786
2-Phenylethanol (Phe)	22,238	193,580***
2-Methylbutanol (Ile)	52,288	63,247*
3-Methylbutanol (Leu)	163,925	222,201**
Butanol	708	901*
Hexanol	2451	2583
Total phenolics (absorbance units)	1.22	1.94*
Tyrosol (mg L^{-1})	14.6	7.0**
Tryptophol (mg L^{-1})	n.d.	13.2***

Results are the mean of three independent replicates. Standard deviations were typically about 10% and never exceeded 20%. In parenthesis, amino acid from which the corresponding volatile compound is derived from in the Ehrlich pathway

n.d. not detected

$P < 0.05$*; $P < 0.01$**; $P < 0.001$***

Tryptophol was only detected in wines made with AWRI2940 (13.2 mg L^{-1}), while tyrosol was significantly lower in wines made with AWRI2940.

Formal sensory analysis was performed on the pilot-scale wines (Fig. 3). ANOVA showed that only four of the 24 sensory attributes assessed differed significantly between the strains. The largest and most notable difference between the strains was for the 'floral' aroma attribute, which was rated significantly higher by the panel ($P < 0.001$) for wines made with strain AWRI2940. There were no significant differences in any other aroma attributes, indicating the relative specificity of impact AWRI2940 had upon wine aroma. Amongst palate terms, 'floral' flavour tended to be rated higher in the AWRI2940 strain, but this was not significant across the panels ($P < 0.2$). On the other hand, wines made with AWRI2940 were rated significantly higher than the control strain for 'astringency' and 'bitter' taste ($P < 0.05$), as well as the visual term 'yellow' colour ($P < 0.001$).

Sequence analysis of the selected strains

In order to identify causative mutations for the 2-PE/2-PEA overproduction phenotype, genome sequencing was performed for ten mutants that were representative of each selection strategy (OFP vs. PFP, EMS-mutagenised vs. spontaneous). Non-mutagenised strains were found to have 80 SNPs on average relative to AWRI796 (see Table S2 for locations), and all harboured mutations in either *ARO4* or *TYR1*. Chemically mutagenised strains presented a more complex picture, with an average of 450 SNPs relative to AWRI796. Nevertheless, all carried mutations in either *ARO4* or *TYR1*. These genes encode for proteins involved in the aromatic amino acid biosynthetic pathway and are under feedback inhibition by the end products of the pathway. *ARO4* encodes a 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthase and catalyses the first step of the pathway, and its activity is under feedback inhibition by L-tyrosine. *TYR1*, which encodes prephenate dehydrogenase, is involved in tyrosine biosynthesi, and its expression is dependent on L-Phe levels (Mannhaupt et al. 1989). All six PFP-resistant colonies and one of the four OFP-resistant colonies sequenced showed different mutations in *TYR1*, while we found different mutations in *ARO4* in the other three OFP-resistant colonies. In eight of the selected strains, the mutations are present in homozygosity, while in the two remaining strains, mutations are in heterozygosity (AWRI2936 for *TYR1* and AWRI2967 for *ARO4*) (Table 3). It is worth noting that recently described alleles of *TOR1* and *FAS2* that confer increased production of 2-PEA in a brewing yeast background (Trindade de Carvalho et al. 2017) were not observed in any of the sequenced strains described here, although one of the OFP mutagenised strains (AWRI2967) showed a mutation in *TOR1* in heterozygosity ($Tor1p^{S2244N}$).

To prove whether mutations in *ARO4* and *TYR1* were causative of the 2-PE/2-PEA overproduction phenotype, allele swaps of six of the mutant alleles (three for the *ARO4* gene and three in the *TYR1* gene) were introduced in the haploid wine strain AWRI1631. All six alleles enhanced resistance to the toxic analogue PFP from the wild-type AWRI1631 baseline of 20 to at least 500 mg L^{-1} (Table 4). These modified strains were then used to ferment synthetic grape juice, and in all cases, we observed an increase in the levels of both 2-PE

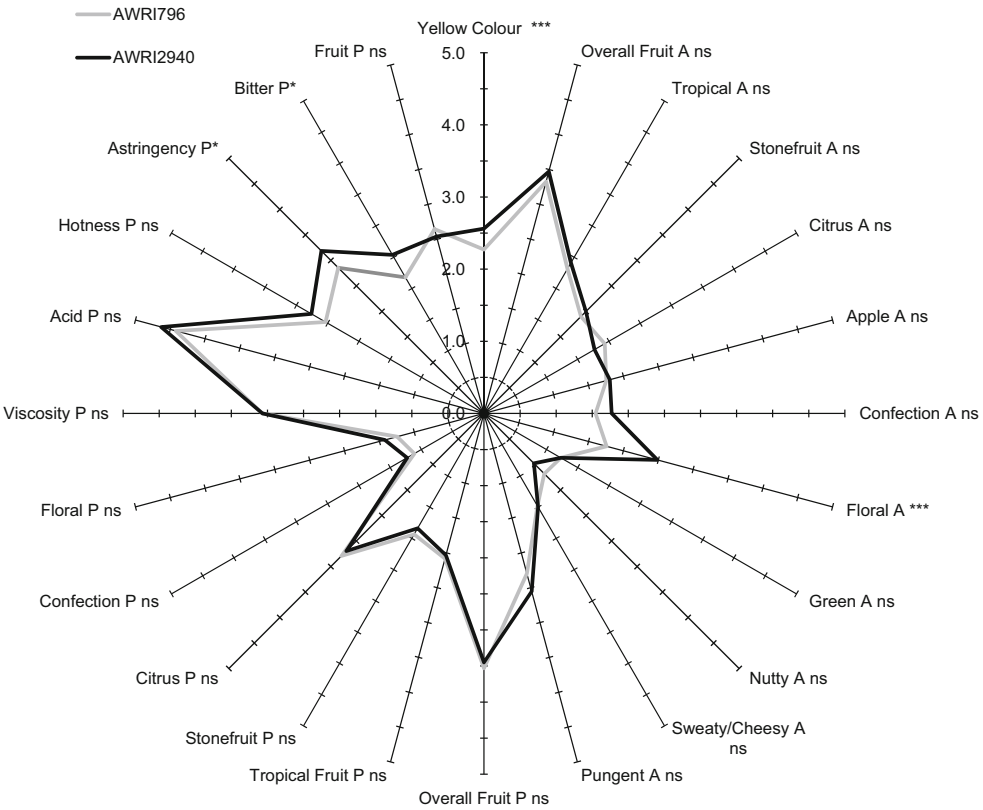

Fig. 3 Mean ratings for aroma (A), palate (P), and colour attributes for the Chardonnay wines (19 L) produced using the parent strain AWRI796 (grey) and the spontaneous PFP-resistant strain AWRI2940 (black) in three fermentation replicates, and assessed by ten judges. *ns* not significant, $P < 0.05$*, $P < 0.01$**, $P < 0.001$***

and 2-PEA at the end of fermentation. All the three *ARO4* allele swaps behaved similarly and resulted in an approximately 20-fold increase in the levels of each 2-PE and 2-PEA when compared with the control strain AWRI1631. The impact of the *TYR1* allele swaps was even greater than those observed for *ARO4*. In wines produced with both Tyr1-2938 and Tyr-2940 strains, we observed a 100-fold increase on the levels of 2-PEA and around 50-fold for 2-PE. As observed previously for the AWRI796 *TYR1* mutants in Chardonnay juice, all the three *TYR1* swaps also resulted in a more substantial increase in the levels of 2-PEA relative to 2-PE (2:1 ratio).

Table 3 Mutations found in genes involved in the aromatic amino acid biosynthetic pathway in the 2-phenylethanol (2-PE)-overexpressing strains

Yeast strain (AWRI number)	Selection	Mutations[$]	*P* value[#]	2-PEA production*	2-PE production*
2936	PFP spontaneous	*TYR1* 323G>R (C108Y)[†]	$3.1E^{-09}$	9.8	4.7
2938	PFP spontaneous	*TYR1* 550G>A (A184T)	$8.8E^{-19}$	16.3	6.6
2940	PFP spontaneous	*TYR1* 591G>T (M197I)	$3.9E^{-11}$	14.4	6.6
2941	PFP spontaneous	*TYR1* 1124C>T (A375V)	1	15.2	5.9
2946	PFP (EMS mutagenesis)	*TYR1* 629C>T (T210I)	$2.5E^{-11}$	17.6	5.6
2947	PFP (EMS mutagenesis)	*TYR1* 562G>A (A188T)	$1.2E^{-12}$	12.2	5.9
2964	OFP spontaneous	*ARO4* 64G>T (D22Y)	$1.4E^{-10}$	4.1	15
2965	OFP spontaneous	*ARO4* 497A>G (Q166R)	$8.7E^{-12}$	4.2	5.9
2967	OFP (EMS mutagenesis)	*ARO4* 584C>Y (S195F)[†]	$1.7E^{-05}$	2.3	4.6
2968	OFP (EMS mutagenesis)	*TYR1* 72G>A (M24I)	$7.8E^{-11}$	7.9	8.5

[†] Mutations in heterozygosity

[$] The amino acid change is shown in parenthesis

*Production of 2-phenylethanol (2-PE) and 2-phenylethyl acetate (2-PEA) in 200 mL Chardonnay ferments, expressed as the fold change relative to the parent strain AWRI796. The control AWRI796 strain produced 73,162 ± 3899 and 893 ± 26.1 μg L^{-1} of 2-PE and 2-PEA, respectively

[#] Significance of the mutation read count versus expected baseline error

Table 4 Characteristics of strains derived from the haploid strain AWRI1631 with chromosomal integrations of the mutated *TYR1* and *ARO4* alleles

Yeast strain	Mutations[$]	Resistance to PFP (mg L^{-1})	2-PEA production*	2-PE production*
AWRI1631		20	1(101 μg L^{-1})[d]	1(11,572 μg L^{-1})[c]
Tyr1-2938	*TYR1* 550G>A (A184T)	>500	105[a]	53[a]
Tyr1-2940	*TYR1* 591G>T (M197I)	>500	109[a]	46[a]
Tyr1-2946	*TYR1* 629C>T (T210I)	>500	61[b]	31[b]
Aro4-2964	*ARO4* 64G>T (D22Y)	500	18[c]	19[b]
Aro4-2965	*ARO4* 497A>G (Q166R)	500	19[c]	20[b]
Aro4-2967	*ARO4* 584C>Y (S195F)	500	22[c]	19[b]

*Production of 2-phenylethanol (2-PE) and 2-phenylethyl acetate (2-PEA), in 200 mL of a synthetic grape juice medium, is expressed as the fold change relative to the parent strain AWRI1631. Standard deviations were typically 10% and never exceeded 20%. Means with the same superscript letter are not significantly different form each other (Tukey's test, $P < 0.05$)

[$] The amino acid change is shown in parenthesis

Discussion

In this study, we successfully isolated a range of mutants derived from the commercial wine strain AWRI796 that overproduce the 'rose' aroma compounds 2-PE and 2-PEA during wine fermentation. Our success in isolating spontaneous mutants, which behave similarly to their mutagenised counterparts in terms of 2-PE and 2-PEA production, makes them superior candidates for commercial application. Genome sequencing of these variants confirmed that, on average, the spontaneous mutants contain six times fewer mutations than the chemically mutagenised strains (80 vs 450 mutations, respectively). This greatly decreases the likelihood of unanticipated or negative effects on fermentation performance and organoleptic properties of wines produced with these strains.

The net compositional-impact of these strains was not restricted to overproduction of 2-PE and 2-PEA. A range of related products derived from the Ehrlich pathway were also found at elevated levels, both in the laboratory-scale and pilot-scale Chardonnay wines, though the increases were modest by comparison to 2-PE/2-PEA. These results indicate that the effects of the mutations in both *ARO4* and *TYR1* are not restricted to the metabolism of aromatic amino acids, but might also affect other amino acid biosynthetic pathways. The acetate esters derived from the branched-chain amino acids Val, Ile, and Leu are characterised by 'fruity' and 'banana' aromas (Swiegers et al. 2005), so overproduction of these compounds may add another layer of complexity to the resulting wines. Importantly, the concentration of ethyl acetate, which is associated with 'nail polish' aroma, was not affected by any of the 2-PE/2-PEA-overproducing strains. Non-specific impacts on flavour metabolite production were also noted by Trindade de Carvalho et al. (2017), where a double *TOR1 FAS2* allele replacement strain significantly increased 2-PEA concentration in beer by approximately twofold, but also significantly increased levels of isobutanol, isoamyl alcohol, and isoamyl acetate.

As anticipated, the 2-PE/2-PEA overproduction phenotype translated into a significant increase in the 'floral' aroma attribute in the pilot-scale Chardonnay wines fermented by spontaneous mutant AWRI2940. These wines were re-analysed 12 months after the end of alcoholic fermentation (approximately 6 months after the formal sensory assessment), and while the levels of 2-PEA had halved in both the control and AWRI2940 wines, the concentrations of 2-PE remained constant (Fig. S3).

Less clear are the drivers for perceived increases in 'astringency' and 'bitterness' palate terms for the Chardonnay wines fermented with AWRI2940, or the slight increase in 'yellow' colour. The total phenolic content of the Chardonnay wines fermented with AWRI2940 was higher than that of the control strain, which may be in part due to accumulation of the phenolic higher tryptophol. Both tryptophol and tyrosol have been associated with 'bitterness' in wine, sake, and beer (Jackson 2014; Soejima et al. 2012; Szlavko 1973). Interestingly, tyrosol and tryptophol are bioactive compounds of pharmaceutical interest showing anticarcinogenic, cardiopreventive, and even antimicrobial properties against pathogenic bacteria (Covas et al. 2003; Cueva et al. 2012; Morshedi et al. 2007). The observed colour difference may also be related to these compounds, though there have been no reports to our knowledge on the specific effect of these compounds on white wine colour. Future research will be conducted to confirm the role of phenolic higher alcohols in bitterness in white wines.

In addition, further research will be necessary to determine wine styles that most benefit from the observed increase in 'rose' and 'floral' aromas achieved by fermentation with AWRI2940. While it has been shown that 2-PE can be an important contributor to the aroma of Pinot Noir (Fang and Qian 2005), Merlot, and Cabernet Sauvignon (Gürbüz et al. 2006), other research has shown that concentrations of 2-PE and 2-PEA are lower in white wines than in red wines. In white varietals, the concentration of 2-PEA is usually below

its odour threshold value (Vilanova et al. 2013). Therefore, the effect of fermentation with AWRI2940 may be more accentuated in white wines, particularly in less aromatic varieties such as Chardonnay, Pinot Gris, or Riesling. This is consistent with results of our pilot-scale winemaking trial in Chardonnay, where the levels of 2-PEA produced by the commercial strain AWRI796 were found to be below its odour threshold, while in wines fermented with AWRI2940, a dramatic increase in 2-PEA levels was observed, well above its odour threshold.

To identify causative mutations for the 2-PE/2-PEA-overproducing phenotype, a whole-genome sequencing approach was used. In three of the four OFP-resistant strains sequenced, we found mutations in the DAHP synthase *ARO4* (Aro4p^{D22Y}, Aro4p^{Q166R}, and Aro4p^{S195F}), which is under feedback inhibition by tyrosine. These three amino acids have been described as playing a role in tyrosine regulation of Aro4p, and mutations in two of these amino acids (Aro4p^{Q166E} and Aro4p^{S195A}) render the enzyme as feedback insensitive by tyrosine, while still retaining a substantial amount of DAHP activity (Hartmann et al. 2003).

In a previous study, a haploid OFP-resistant laboratory strain with a mutation in *ARO4* was isolated (Fukuda et al. 1991) which was found to overproduce 2-PE and accumulated both L-Phe and L-Tyr intracellularly. This haploid strain harboured a mutation in the same position (Aro4p^{Q166K}) to that found in homozygosity in our OFP-resistant strain AWRI2965 (Aro4p^{Q166R}), and it was found to be free of feedback inhibition from tyrosine (Fukuda et al. 1992a). In both cases, the neutral amino acid Gln in position 166 was mutated to a basic amino acid. When we introduced the Aro4p^{Q166R} mutation in a haploid wine strain background, we also observed an intracellular accumulation of the aromatic amino acids L-Tyr, L-Phe, and L-Trp after fermentation of a synthetic grape juice (Table S1). It is also worth noting that Aro4p^{Q166K} and other Aro4p mutations are frequently used in synthetic biology studies in order to increase carbon flux into the aromatic amino acid biosynthetic pathway for the overproduction of valuable secondary metabolites such as opioids, polyketides, or vanillin (Galanie et al. 2015; Gold et al. 2015).

Based upon these prior works, we can hypothesise that the mutations found in Aro4p in three of the OFP-resistant strains sequenced (AWRI2964, 2965, and 2967) will render this protein insensitive to feedback inhibition by tyrosine, redirecting pathway flux towards the accumulation of aromatic amino acids and consequent overproduction of 2-phenylethanol through the Ehrlich pathway. The integration of the three mutated alleles of *ARO4* in the haploid wine strain AWRI1631 confirmed that these are the causative mutations for the 2-PE/2-PEA overproduction phenotype of these strains.

All the six PFP-resistant strains that were sequenced showed different mutations in *TYR1*. Previously, an unidentified mutation in *TYR1* in a haploid laboratory strain had been linked with resistance to PFP and 2-PE overproduction (Fukuda et al. 1991a). This mutation led to a decrease in prephenate dehydrogenase activity, encoded by *TYR1*, while the activity of DAHP was found to be markedly increased. In addition, this strain also produced lower amounts of L-Tyr and higher concentrations of both L-Phe and L-Trp. When we introduced one of the *TYR1* mutations (Tyr1-2940) in a haploid wine strain background, we observed a similar result; this strain accumulated L-Phe in the fermentation media (extracellularly), and the levels of L-Tyr were reduced intracellularly.

In this context, it is interesting to note that in both AWRI796 and AWRI1631 backgrounds, mutations in *TYR1* seem to have a greater impact upon the overall formation of fusel alcohols than those in *ARO4*. Fukuda et al. (1990b) also described that mutations strains selected in PFP had a greater impact than those selected in OFP for 2-PE.

Mutations in both *ARO4* and *TYR1* are likely dominant, since we were able to isolate strains (AWRI2967 and AWRI2936) resistant to both toxic analogues of phenylalanine with heterozygous mutations for both genes. Supporting this, *ARO4* has been used as a dominant selection marker in yeast centromeric vectors for the transformation of industrial strains of *S. cerevisiae* (Fukuda et al. 1992b). We also observed a dosage effect in the formation of 2-PEA by mutations in both *ARO4* and *TYR1*. Strains with heterozygous mutations in *ARO4* and *TYR1* produced half as much 2-PEA as the strains with homozygous mutations in the same genes. The exact mechanism leading to the formation of homozygous mutations (loss of heterozygosity) in these genes is unknown. It is unlikely that this is a direct result of the mutagenesis procedure, but is probably due to a mitotic gene conversion event of the wild-type allele by the mutated allele after selection pressure is applied. In the process of isolating resistant strains to toxic analogues of L-Phe, single colonies were streaked several times in increasing concentrations of these selective agents to make sure that we were obtaining stable mutants.

The various yeast strains isolated in this study provide a novel tool for winemakers to modulate wine style to predetermined marker specifications. The strains, along with characterised mutant *ARO4* and *TYR1* alleles, also have the potential to be used as cell factories for the production of compounds of biotechnological interest. Current production processes for 2-PE involve chemical synthesis, yet it is widely used in cosmetics, perfumery, and food industries, where the marketplace increasingly demands natural methods of production.

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Compliance with ethical standards

Competing interests The authors declare that they have no conflicts of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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