PICFPA	SAAPPA / SASPA / SAT	DFTS	Winetech
Canning Fruit Producers' Assoc.	Fruitgro Science	Dried Fruit Technical Services	
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Indicate (X) client(s) to whom this final report is submitted.
Replace any of these with other relevant clients if required.

FINAL REPORT
FOR 2012

PROGRAMME & PROJECT LEADER INFORMATION

	Programme leader	Project leader
Title, initials, surname	Prof FF Bauer	Drs PR Young and Debra Rossouw
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PROJECT INFORMATION

Project number	IWBT Y08/07	
Project title	Metabolic engineering of yeast to enhance flavour and aroma development during wine fermentation	
Project Keywords		

Industry programme	**CFPA**	
	Deciduous	
	DFTS	
	Winetech	✓ Microbiology committee
	Other	

Fruit kind(s)	
Start date (dd/mm/yyyy)	2008
End date (dd/mm/yyyy)	2011/2012

(Note: adjust footer – insert the project number no, researcher and research institution)

FINAL REPORT

(Completion of points 1-5 is compulsory)

1. Executive summary

Give an executive summary of the *total* project in no more than 250 words

The specific objectives of the project are to express grapevine-derived genes encoding for enzymes involved in the formation of important flavour/aroma compounds in wine, in yeast. The recombinant yeast will be evaluated for their ability for the *de novo* production of flavour/aroma compounds.

Three genes with the potential to form flavour/aroma compounds have previously been isolated and characterised from grapevine (reported in Winetech 5-09A – 2005-2007). These genes can be grouped into two tasks for this application: (1) the terpene synthases/cyclases (TPS) and (2) the carotenoid cleavage dioxygenases (CCD). The selected genes have been cloned into yeast expression vectors and transformed into three yeast strains for evaluation (volatile flavour/aroma formation). Yeast genes that could potentially affect the production of the flavour/aroma compounds in yeast have subsequently been identified and targeted for genetic modification (i.e. overexpression or knock-out). These genes have been cloned into yeast expression vectors and transformed, in various combinations, into yeast strains co-expressing grapevine terpene synthases. The resultant recombinant yeast strains were evaluated for their volatile flavour/aroma production.

Additional work on metabolic regulation in yeast has led to the establishment of semi-predictive metabolic models which can be used to design improved strategies for enhancing aroma production in yeast. These models were developed in projects within the NRF funded wine science research niche area. To apply this newly gained knowledge, the project had added new milestones for 2011 (see task 3, milestones 2-5). These have led to a better understanding of regulatory networks responsible for aroma production.

2. Problem identification and objectives

State the problem being addressed and the ultimate aim of the project.

Flavour and aroma compounds present in grapes play a major role in the ultimate quality of the wine. These flavour and aroma compounds are generally cultivar-specific and are known to contribute to the varietal character of the cultivars. These compounds are influenced by the macro- and microclimate of the vineyard and can be managed viticulturally. These compounds, that include monoterpenes, sesquiterpenes and norisoprenoids, are formed exclusively in the plant and are extracted from the grapes during the wine making process.

Following on the isolation and characterisation of genes involved in the carotenoid metabolic pathway from grapevine (reported in Winetech 5-09A titled “Isolation and characterisation of carotenoid pathway genes and promoters from *Vitis vinifera* as resources towards stress-tolerant grapes with superior quality” – 2005-2007); three genes with the potential to form flavour/aroma compounds have already been identified, isolated, cloned and sequenced. These genes can be grouped into two tasks for this application: (1) the terpene synthases/cyclases (TPS) and (2) the carotenoid cleavage dioxygenases (CCD).

The aim of this project is to identify and isolate genes that potentially play a role in the formation of flavour and aroma compounds, and generate recombinant yeast expressing these genes. The recombinant yeast will subsequently be evaluated for their potential for *de novo* synthesis of flavour and aroma compounds.

The extended work-plan of the project approved in 2010 for 2011 (see TASK 3) also aims at providing novel tools to link specific yeast strains with specific aroma production profiles and to provide a predictive capability that will allow a better and more targeted selection of yeast strains for specific conditions and grape musts.

Furthermore, a genetic screen for mutants with changed aromatic profiles was investigated (Styger et al. 2011).

3. Workplan (materials & methods)

Project number / researcher / research institution

List trial sites, treatments, experimental layout and statistical detail, sampling detail, cold storage and examination stages and parameters.

TASK 1-2

Milestone 1

Cloning of the candidate genes into appropriate yeast expression vectors and transformation into *Saccharomyces cerevisiae* for evaluation.

The candidate genes (*VvTPS2*, *VvTPS3* and *VvCCD1)* will be cloned into yeast integration cassettes under control of the constitutive yeast phosphoglycerate kinase (*PGK1*) promoter. The cassettes will be integrated into the *ura3* locus of laboratory *Saccharomyces cerevisiae* strains: (1) BY4742, (2) Σ1278b, (3) W303a, and (4) JRY7179 (a *upc2-1* mutant of W303a).

Milestone 2

Selection and genetic verification of the putative transformants.

Putative transformants will be selected based on uracil auxotrophy. Integration of the expression cassette into the *ura3* locus of the genome will be verified using Southern hybridisations. Expression of the transgenes of interest will be verified by northern hybridisations.

Milestone 3

Identification and quantification of the volatiles formed by the recombinant yeast.

For this task the *VvTPS3* (monoterpene synthase) was used to setup the respective analytical "pipeline". The volatiles formed by the recombinant yeast were isolated using Solid Phase Micro-Extraction (SPME) and/or liquid extaction (LE). The separation, identification and quantification were performed using GC/MS (for HS-SPME) and GC-FID (for LE). Individual compounds were quantified using an authentic standard (α-terpineol). The volatiles formed by the recombinant strains will be compared to the volatiles formed by untransformed yeast as well as a strain containing the *PGK1* promoter expression cassette, without any gene being expressed.

Milestone 4

Optimising the levels and relative amounts of the volatiles formed during alcoholic fermentation.

Milestone 5 [2008-2011]

The availability of the grapevine genome sequence in 2007 has facilitated the identification of a number of additional candidate genes (TPS- and CCD-encoding genes). Additional putative TPSs and CCDs have been identified in the grapevine genome and are currently being targeted for isolation and characterisation.

Milestone 6 [2011, July]

Additional genes from Milestone 5 are evaluated (same workplan: Milestone 1-4)

TASK 3

Screening of publicly accessible sequence databases for additional genes (from yeast or grapevine) encoding enzymes that are potentially involved in the formation of flavour and aroma compounds will continue throughout the project.

Milestone 1 Data mining information from platform technologies (e.g. transcriptomic and metabolomic data) are proving useful in identifying endogenous yeast genes that potentially play a role in flavour and aroma production (in yeast). Transcriptomic profiling of yeast strains in conjunction with aroma profiling has already generated data that can be incorporated into semi-predictive metabolic models (Rossouw et al., 2008). Models generated in this way have proven useful, for example, predicting the impact of single gene overexpression on the concentration of aroma compounds produced in yeast.

Milestone 2 [2010-ongoing]

Modelling of gene expression and volatile aroma compounds and identification of target genes for aroma profile modification.

Milestone 3 [2010, July onwards]

Identification of target genes and planning of cloning and transformation strategies.

Milestone 4 [2010, December onwards]

Cloning of target genes and vector construction for overexpression in selected yeast strains.

Milestone 5 [2011, July onwards]

Evaluation of transformants in small scale fermentations. HPLC and GC-FID profiling analysis of the yeast exometabolome.

Availability of equipment/infrastructure/resources:

The *VvTPS2*, *VvTPS3* and *VvCCD1* genes and yeast strains for the project are already available. The yeast transformation, selection and genetic analysis are routine techniques in our environment. Sequencing can be performed at the Central Analytical Facility (CAF) of Stellenbosch University (SU). GC-MS profiling technologies will be used for the volatile screening within the GC-MS unit of CAF at SU. The IWBT also has an in-house chemical analytical facility with trained staff, one of which will be involved in the method development and validation on the GC-MS methods. The CAF of SU can also provide instrument time, expertise and student training.

4. Results and discussion

State results obtained and list any benefits to the industry. Include a short discussion if applicable to your results.
This final discussion must cover ALL accumulated results from the start of the project, but please limit it to *essential* information.

Seventeen putative terpene synthase (TPS) and four putative cleavage dioxygenases (CCD) encoding genes have been identified by orthologue searches in the grapevine genome (Figure 1)
Seven putative terpene synthase (TPS) encoding genes and three carotenoid cleavage dioxygenases (CCD) have been subsequently isolated, sequenced and cloned into yeast and bacterial expression vectors, respectively

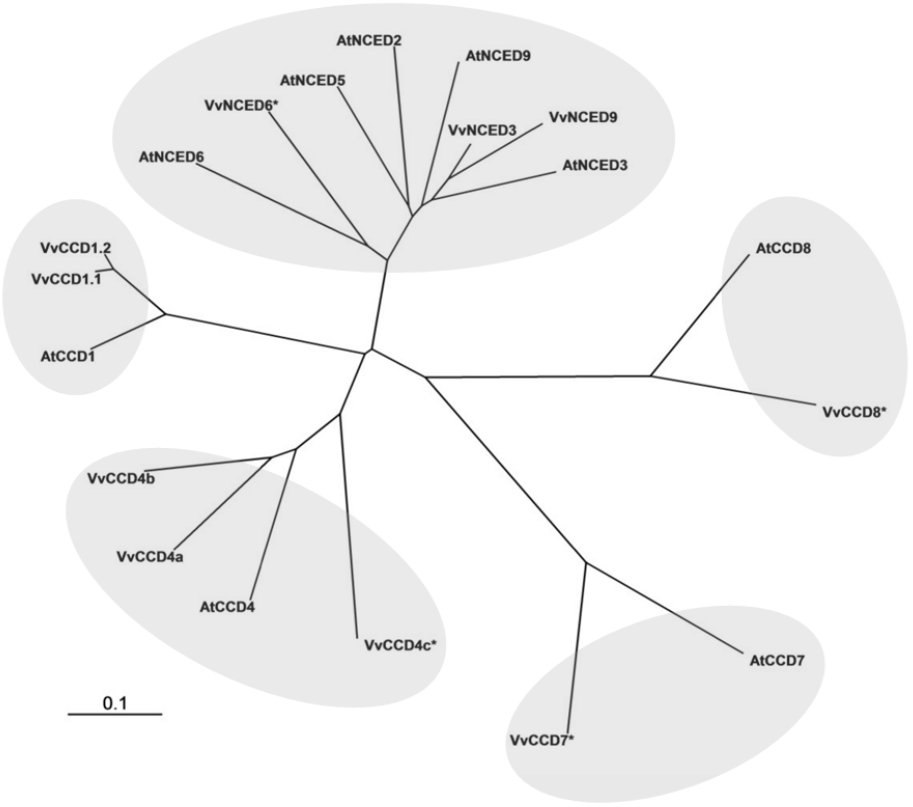

Figure 1. Phylogenic tree of *Arabidopsis thaliana* and grapevine CCDs. Based on protein similarity this tree shows the various clades of the CCD protein family. Proteins indicated with * have not been isolated and are based on sequence prediction software. The NCED clade is added for completeness.

Three additional genes (one from grapevine [*VvGPS1*] and two from *Saccharomyces cerevisiae [tHMG1* and *IDI1]*) have been targeted to increase flux to monoterpene production.
A monoterpene synthase (*VvTPS03*) has been shown to be functional and produced (-)-terpinen-4-ol in the yeast system (Figure 2 A and B)
Expression of two of the additional three genes investigated (*tHMG1* and *IDI1*) that were expressed to improve flux to monoterpene production in yeast, led to a 8-fold increase in the (-)-terpenin-4-ol levels in yeast relative to the *VvTPS03* expressing strain (Figure 2).

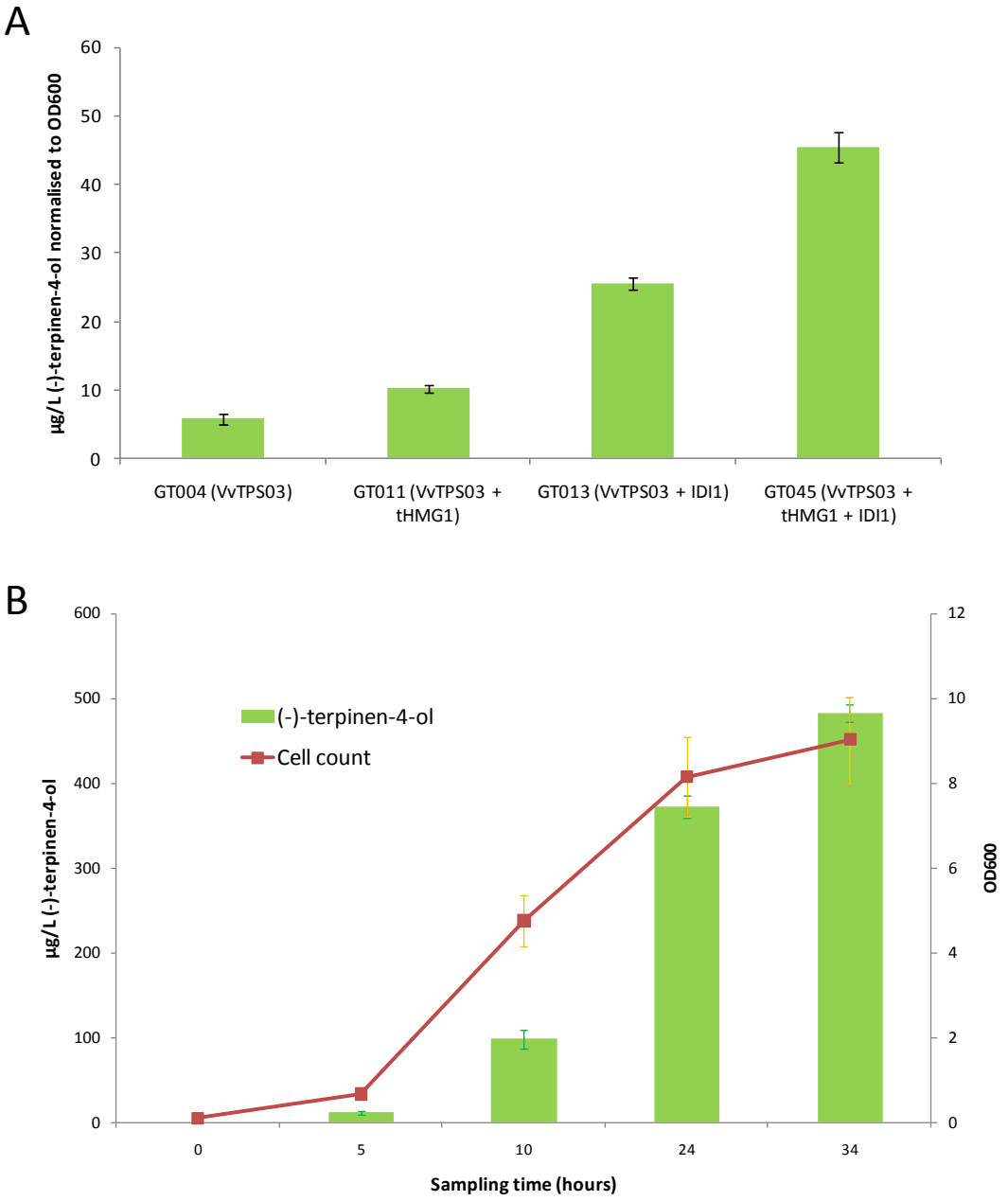

Figure 2. (-)-terpenin-4-ol production in the recombinant *Saccharomyces cerevisiae* W303-1a yeast strains. (-)-terpinen-4-ol levels are normalised to OD_{600} measurement and the internal standard, 2,6-dimethyl-hepten-2-ol. Sampling at 24 hours (A) The GT045 strain was used to determine terpene production over time (B) Each bar represents the average of three biological repeats.

Figure 3. Terpin-4-ol production in the recombinant *Saccharomyces cerevisiae* JRY7179 yeast strains. Values are normalised to OD600 and the internal standard (3-octanol). Values are expressed as percentages relative to the first sampling point (20 hours). Each bar represents the average of three biological repeats.

The optimised yeast expression system and the chemical analytical methods (SPE-GC-FID and HS-SPME-GC/MS) have provided a "pipeline" to functionally characterise additional putative terpene synthase encoding genes.

Saccharomyces cerevisiae do not produce carotenoids, and recombinant yeast producing specific carotenoids are currently limited. The functionality of the CCDs was consequently evaluated in an available bacterial (*Escherichia coli*) system where various carotenoid substrates (for carotenoid cleavage dioxygenases) are produced. The CCD1, CCD4a and CCD4b were shown to cleave carotenoids in the heterologous bacterial system to form volatile apocarotenoids (norisoprenoids). Figure 4 and Table 1 summarise the results. The results show that CCDs cleave specific carotenoids to form the corresponding volatile C_{13} cleavage product (norisoprenoid).

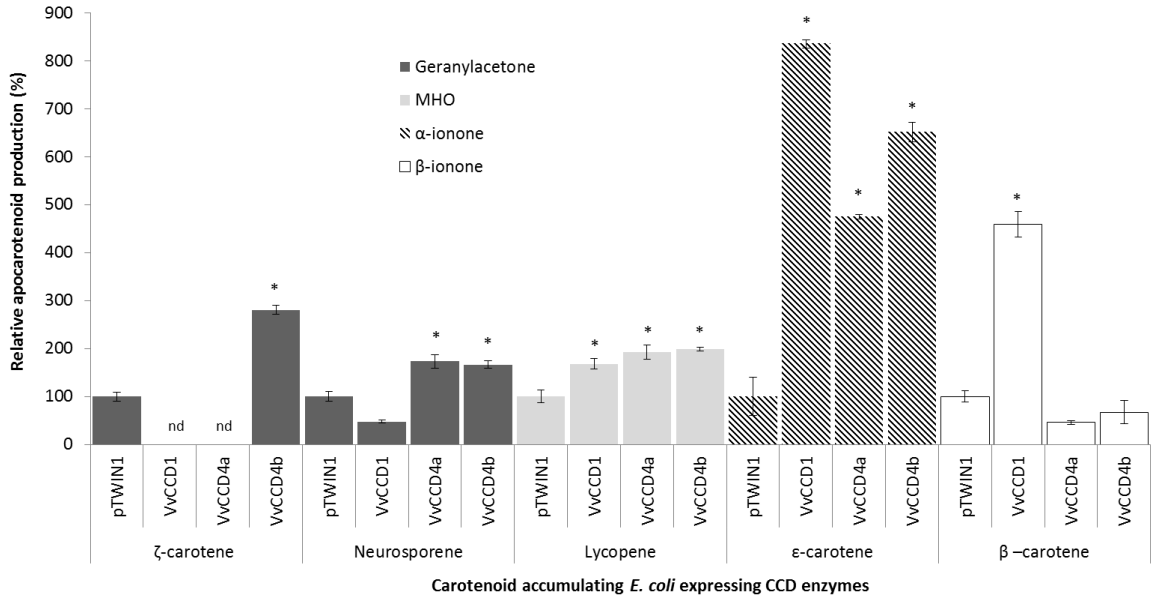

Figure 4. Functionality and substrate specificity of VvCCD1, VvCCD4a and VvCCD4b in a heterologous *in vivo* bacterial system. CCDs were expressed in *Escherichia coli* engineered to accumulate specific carotenoids. Carotenoids produced before cleavage were determined using UPLC. Volatile apocarotenoids produced after cleavage were determined using GC-MS.

Table 1. **Functionality and substrate specificity of VvCCD1, VvCCD4a and VvCCD4b in a heterologous *in vivo* bacterial system**. CCDs were expressed in *Escherichia coli* engineered to accumulate specific carotenoids. Carotenoids produced before cleavage were determined using UPLC. Volatile apocarotenoids produced after cleavage were determined using GC-MS.

Carotenoid accumulating plasmid (pAC-)	**Carotenoid → Apocarotenoid**	**Cleavage position**	**pTWIN1 (vector control)**	**VvCCD1**	**VvCCD4a**	**VvCCD4b**
			Average ng.L^{-1} apocarotenoid produced ± standard deviation (n=3)			
pAC-ZETA	ζ-carotene (80%)[1] → Geranylacetone	9,10(9',10')	803.61±75.20	ND[2]	ND	2255.04±74.57
pAC-NEUR	Neurosporene (100%)[1] → Geranylacetone	(9',10')	388.61±12.73	184.18±53.08	671.43±32.15	646.39±37.01
pAC-LYC	Lycopene (100%)[1] → 6-MHO	5,6(5',6')	393.19±49.42	660.70±44.36	756.00±59.27	782.00±15.45
pAC-EPSILON	ε-carotene (70%)[1] → α-ionone	9,10(9',10')	40.24±8.35	336.15±16.32	190.91±3.57	262.08±1.88
pAC-BETA	β –carotene (100%)[1] → β-ionone	9,10(9',10')	76.28±8.47	349.61±20.46	< LOQ[30]	< LOQ

[1] The percentage of the specific carotenoid substrate present in the strain before VvCCD induction as determined by UPLC analysis
[2] "ND" Not detected
[3] "< LOQ" Below level of quantification

The optimised yeast expression system and the chemical analytical methods (HS-SPME-GC/MS) have provided a "pipeline" to functionally characterise the additional 6 putative terpene synthase encoding genes.

Amended workplan in 2011:

In the amended workplan for 2011, various aroma-relevant pathways were analysed ojn a genetic and metabolic level.
This work led to the identification of the major drivers of aroma production by wine yeast strains, and a predictive model of how specific must composition will lead to specific aromatic features in the wine. These findings were published (Rossouw et al. 2012; Styger et al. 2011; Jain et al. 2011).
Importantly, the findings for the first time clearly demonstrate the importance of redox balancing, as measured by the ratio of NAD^+ on NADH, on the production of secondary aroma metabolites, a link that had not been demonstrated before.
As an example of the data, Figure 3 below summarises many of the findings, and shows the impact of specific double or triple mutants on the production of specific aromatic compounds when compared to the parental wild-type. These changes are shown as fold changes (From Styger et al. 2011).

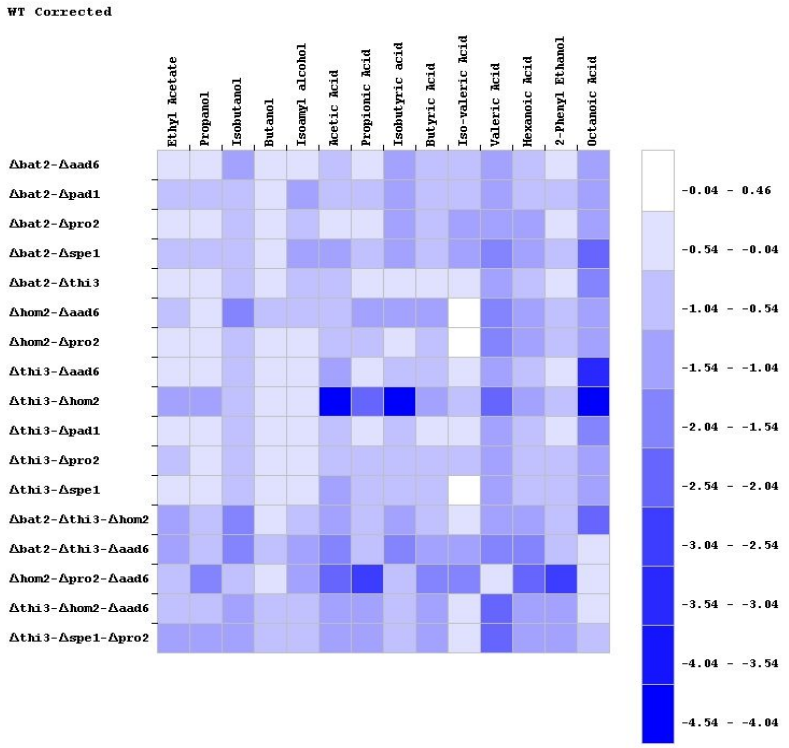

Figure 5. Fold changes of aroma compounds produced by several double or triple mutants strains when compared to wild-type. The colour intensity indicates the fold change as shown in the figure.

Milestone	**Achievement**
1. Cloning of the candidate genes into appropriate yeast expression vectors and transformation into *Saccharomyces cerevisiae* for evaluation	The *VvTPS2*, *VvTPS3* and *VvCCD1* genes have been cloned into yeast expression vectors.
2. Selection and genetic verification of integration and expression of the putative transformants	The transformants have been genetically characterised for integration (Southern hybridisations and/or PCR) and expression of the transgene (northern hybridisations)
3. Extraction, separation, identification and quantification of the volatiles formed by the recombinant yeast during fermentation (*VvTPS3*, the monoterpenes α-terpineol).	The HS-SPME-GC/MS and GC-FID were evaluated for their suitability for for yeast cultures
4. Optimising the levels and relative amounts of the volatiles formed during alcoholic fermentation	A number of *Saccharomyces cerevisiae* mutants have been identified (from literature) with the potential to improve/increase the production of terpenoids in yeast. These strains have been requested and will be evaluated
5. Additional putative TPSs and CCDs have been identified in the grapevine	17 Additional putative TPS- and 4 additional CCD-encoding genes have been identified in

genome and are currently being targeted for isolation (PCR-mediated) and characterisation	the grapevine genome. Six TPS's and 2 additional CCDs have been isolated.
6. Additional genes from Milestone 5 are evaluated (same workplan: Milestone 1-4)	n/a
Task 3: Milestones	
1. Screening of publicly accessible sequence databases and literature for additional genes (from yeast or grapevine) encoding enzymes that are potentially involved in the formation of flavour and aroma compounds	Two yeast genes (ScIPI and ScHMG1) and a grapevine gene (VvGPS1) are currently being investigated.
2. Modelling of gene expression and volatile aroma compounds and identification of target genes for aroma profile modification.	This milestone has been completed. Herewith the Abstract of a paper published in Genetics (see Outputs): Transcription factors have been proposed as primary targets of evolutionary adaptation. However, there is little concrete evidence for such evolutionary changes. *Saccharomyces cerevisiae* wine yeast strains are a geno- and phenotypically diverse group of organisms that have been selected to produce specific styles of wine. The data suggest that differences in the expression of a limited number of transcriptional regulators may be largely responsible for differences in metabolic phenotypes of such strains, and that the production of aroma-active metabolites of a strain can be shifted in a relatively predictable manner by changing expression levels of individual transcription factors.
3. Identification of target genes and planning of cloning and transformation strategies.	This milestone has been completed: See Styger *et al*, 2011. During alcoholic fermentation many volatile aroma compounds are formed by *Saccharomyces cerevisiae*, including esters, fatty acids and higher alcohols. While the metabolic network that leads to the formation of these compounds is reasonably well mapped, surprisingly little is known about specific enzymes involved in specific reactions, the regulation of the network and the physiological roles of individual pathways within the network. Furthermore, different yeast strains tend to produce significantly different aroma profiles. These differences are of tremendous biotechnological interest, since producers of alcoholic beverages such as wine and beer are searching for means to diversify and improve their product range.

	Various factors such as the redox, energy and nutritional balance of a cell have previously been suggested to directly or indirectly affect and regulate the network. To gain a better understanding of the regulations and physiological role of this network, we screened a subset of the EUROSCARF strain deletion library for genes that, when deleted, would impact most significantly on the aroma profile produced under fermentative conditions. The ten genes whose deletion impacted most significantly on higher alcohol production, were selected and further characterized to assess their mode of action within or on this metabolic network. This is the first description of a large scale screening approach using aroma production as the primary selection criteria, and the data suggest that many of the identified genes indeed play central and direct roles within the aroma production network of *S. cerevisiae*.
4. Cloning of target genes and vector construction for overexpression in selected yeast strains.	On-going in 2012 as part of the new Winetech program. Also see Styger et al. 2011a,b and 2012 (in preparation)
5. Evaluation of transformants in small scale fermentations. HPLC and GC-FID profiling analysis of the yeast exometabolome.	Many strains with mutations that impact on aroma production were assessed (see Styger *et al.* 2011a)

5. Accumulated outputs

List ALL the outputs from the start of the project.
The year of each output must also be indicated.

Technology development, products and patents

Indicate the commercial potential of this project (intellectual property rights or a commercial product(s)).

1. HS-SPME-GC/MS method for extracting and analysing volatiles from yeast cultures. Optimisations have lead to a 2-fold (200%) improvement of monoterpene (α-terpineol) production in the yeast strain evaluated.
2. A yeast strain with improved flux to the isoprenoid/terpenoid pathway should be relevant in a number of applications.
3. The first predictive model for aroma production. The model allows to redict the changes in aromatic profiles associated with genetic perturbations.
4. Identification of genes with most significant impact on higher alcohol production.
5. A significant improvement of our understanding of how must composition and yeast genetic background combine to determine the aroma production potential of individual fermentations.

Human resources development/training

Indicate the number and level (e.g. MSc, PhD, post doc) of students/support personnel that were trained as well as their cost to industry through this project. Add in more lines if necessary.

Project number / researcher / research institution

	Student level (BSc, MSc, PhD, Post doc)	Cost to project (R)
1.	Ewan Potgieter (Hons, 2008)	
2.	Lee-Gavin Williams (Hons, 2010)	
3.	Samantha Dockrall (Hons, 2010)	
4.	Samantha Dockrall (MSc, 2011-ongoing)	
5.	Ilse Bezuidenhout (Hons, 2011)	
6.	Ilse Bezuidenhout (MSc, 2012-ongoing)	
7.	Gustav Styger (PhD, Graduated in 2011)	
8.	Vishist Jain (PhD, graduated in 2010)	
9.	Debra Rossouw (Post-doc)	

Publications (popular, press releases, semi-scientific, scientific)

Peer-reviewed articles

(1) Young P.R., Lashbrooke J.G., Alexandersson E., Jacobson D., Claudio Moser C., Velasco R. & Vivier M.A. (2012) The genes and enzymes of the carotenoid metabolic pathway in Vitis vinifera L. *BMC Genomics*

(2) Rossouw, D., D. Jacobsen & **F.F. Bauer. 2012**. Transcriptional regulation and the diversification of metabolism in wine yeast strains. *Genetics* **190:**251-261.

(3) Styger, G. D. Jacobson & **F.F. Bauer. 2011a.** Identifying genes that impact on aroma profiles produced by *Saccharomyces cerevisiae*. *Applied Microbiology and Biotechnology* **91:**713-730.

(4) Styger, G., B.A. Prior & **F.F. Bauer. 2011b.** Wine Flavor and Aroma: A Review. *Journal of Industrial Microbiology and Biotechnology* **9:**1145-1159.

(5) Jain, V.K., B. Divol, B.A. Prior & **F.F. Bauer. 2012.** Effect of alternative NAD+ regenerating pathways on the formation of primary and secondary aroma compounds in a *Saccharomyces cerevisiae* glycerol defective mutant. *Applied Microbiology and Biotechnology* **93**:131-141.

Full length peer-reviewed conference proceedings

(6) Rossouw, D. & F.F. Bauer. 2010. Comparative omics of wine yeast strains. Internationaler IVIF Kongress Intervitis Interfructa Stuttgart. In: Micro-organisms – alcoholic fermentation. pp 1-10 (on invitation).

(7) Vivier, M.A., M du Toit & F.F. Bauer. 2011. Wine Metabolomics: How yeast and bacteria transform the grape metabolome. Proceedings of 2nd edition of the international conference series on Wine Active Compounds. pp 11-13 (on invitation).

(8) Bauer, F.F. 2011. Yeast – the wine builder. Proceedings of the 34th World Congress of Vine and Wine of the OIV – The construction of wine. Porto, Portugal. pp 1-10 (on invitation).

Presentations/papers delivered

(9) Young, P.R., J.G. Lashbrooke & M.A. Vivier. 2008. Biotechnological potential of carotenoid biosynthetic genes isolated from *Vitis vinifera* L. Cape Biotechnology Forum, Lord Charles Hotel, Somerset West. (30 Nov – 2 Dec)

(10) Lashbrooke, J.G., S.J. Dockrall, P.R. Young & M.A. Vivier. 2012. The Carotenoid Cleavage Dioxygenase (CCD) gene family gene family in *Vitis vinifera* L. South African Association of Botany (SAAB 2012), Pretoria University. (15-18 January)

4. Total cost summary of project

	Year
Total cost in real terms for year 1	
Total cost in real terms for year 2	
Total cost in real terms for year 3	
Total cost in real terms for year 4	
Total cost in real terms for year 5	
TOTAL	

CFPA	Deciduous	DFTS	Winetech	THRIP	Other	TOTAL