RESEARCH ARTICLE

Functional improvement of *Saccharomyces cerevisiae* to reduce volatile acidity in wine

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Abstract

Control of volatile acidity (VA) is a major issue for wine quality. In this study, we investigated the production of VA by a deletion mutant of the fermentation stress response gene *AAF1* in the budding yeast *Saccharomyces cerevisiae*. Fermentations were carried out in commercial Chardonnay grape must to mimic industrial wine-making conditions. We demonstrated that a wine yeast strain deleted for *AAF1* reduced acetic acid levels in wine by up to 39.2% without increasing the acetaldehyde levels, revealing a potential for industrial application. Deletion of the cytosolic aldehyde dehydrogenase gene *ALD6* also reduced acetic acid levels dramatically, but increased the acetaldehyde levels by 41.4%, which is not desired by the wine industry. By comparison, *ALD4* and the *AAF1* paralog *RSF2* had no effects on acetic acid production in wine. Deletion of *AAF1* was detrimental to the growth of *ald6Δ* and *ald4Δald6Δ* mutants, but had no effect on acetic acid production. Overexpression of *AAF1* dramatically increased acetic acid levels in wine in an Ald6p-dependent manner, indicating that Aaf1p regulates acetic acid production mainly via Ald6p. Overexpression of *AAF1* in an *ald4Δald6Δ* strain produced significantly more acetic acid in wine than the *ald4Δald6Δ* mutant, suggesting that Aaf1p may also regulate acetic acid synthesis independently of Ald4p and Ald6p.

Introduction

Control of volatile acidity (VA) is a critical issue for the industrial manufacturing of wine. During wine fermentation, the production of acetic acid, by far the most abundant volatile acid, can have a dramatic effect on the quality of the final product. At levels typically found in wine, 0.2–0.6 g L^{-1}, acetic acid adds a pleasant tartness. Also, it serves as a precursor to acetate esters, which are responsible for the fruity character of many wines. However, at higher levels, acetic acid in wine is generally considered to be a spoilage product; acetic acid production can result in the formation of unpleasant volatile compounds such as ethyl acetate that smells like fingernail polish (Moreno-Arribas & Polo, 2005). In addition to undesirable aromas, high levels of acetic acid are toxic to yeast and may lead to stuck alcoholic fermentations. Three methods have been used by the wine industry to reduce acetic acid levels in wine (Vilela-Moura *et al.*, 2011): (1) blending wine with high VA with low VA wine; this, however, often leads to a reduction in the quality of wine; (2) reverse osmosis, which is expensive and does not significantly remove ethyl acetate; (3) refermentation using additional yeast strains (Vilela-Moura *et al.*, 2008); this process consists of mixing acidic wine with musts from freshly crushed grapes and inoculation of oxidatively growing yeasts which can use acetic acid as a carbon source. This practice, however, makes wine prone to contamination and may have a detrimental impact on wine. Therefore, alternative methods of controlling volatile acidity have been investigated in recent years. Instead of removing acetic acid from wine, new strategies focused on reducing the formation of acetic acid during fermentation. One example of this strategy is the use of mixed *Saccharomyces* and non-*Saccharomyces* strains in fermentations. Strains of *Torulaspora delbrueckii* and *Candida zemplinina* have been combined with *Saccharomyces cerevisiae*, and a 50–53% reduction in volatile acidity has been obtained (Bely *et al.*, 2008; Renault *et al.*, 2009; Rantsiou *et al.*, 2012). In a recent study, Cor-

dente *et al.* (2013) used a classical mutagenesis approach to isolate cerulenin-resistant strains from a diploid commercial wine yeast that produced less acetic acid during wine fermentation.

Evidence from experiments in laboratory media and synthetic grape must with yeast carrying deletion mutants has shown that acetate is produced mainly by the cytosolic acetaldehyde dehydrogenase Ald6p and subtly by a mitochondrial route involving Ald5p (Saint-Prix *et al.*, 2004). The other acetaldehyde dehydrogenases in yeast, the mitochondrial form Ald4p, and the minor cytosolic forms Ald2p and Ald3p have no effects on acetic acid levels (Remize *et al.*, 2000). However, in yeast cells where all known *ALD* genes have been completely eliminated, acetic acid is still produced, suggesting alternative pathways during fermentation (Saint-Prix *et al.*, 2004).

We have recently demonstrated that the fermentation stress response (FSR) gene YML081W/*AAF1* regulates acetic acid production in standard laboratory growth conditions (Walkey *et al.*, 2012). *AAF1* encodes a protein that contains a C2-H2 zinc-finger domain at the N-terminus, and this protein is a potential transcription factor (Badis *et al.*, 2008). Null mutants in the standard S288C laboratory strain displayed sensitivity to osmotic stress (Yoshikawa *et al.*, 2009), nickel sulfate (Arita *et al.*, 2009), and topoisomerase damage (Reid *et al.*, 2011). Previous high-throughput studies have identified this gene encoding a nuclear protein (Huh *et al.*, 2003) that shares 38.0% identity and 54.5% similarity in the entire sequence, as well as 80.0% identity in the N-terminal zinc-finger domains with its paralog, Rsf2p/Zms1p, that arose from the whole genome duplication (Byrne & Wolfe, 2005). Rsf2p/Zms1p is a transcription factor that governs expression of genes required for glycerol-based and respiratory growth (Lu *et al.*, 2005). Moreover, Grabowska & Chelstowska (2003) showed that Rsf2p may regulate the expression of *ALD6*. We have shown that *AAF1* regulates expression of *ALD4* and *ALD6* (Walkey *et al.*, 2012). In this study, we investigated how the deletions of *AAF1*, *RSF2*, *ALD4* and *ALD6* in wine yeast affect the production of acetic acid during Chardonnay grape must fermentation. Here, we show that deletion of *AAF1* reduced acetic acid levels by up to 39.2% without increasing the acetaldehyde concentration in the wine, revealing a potential industrial application.

Materials and methods

Yeast strains and culture conditions

Yeast strains used in this study are described in Table 1. All strains were derived from Enoferm M2, a widely

Table 1. Yeast strains in this study

Name	Genotype
M2	*MATα/a*
aaf1Δ	*MATα/a, aaf1::kanMX4/aaf1::kanMX4*
aaf1Δ/AAF1-GFP	*MATα/a, aaf1:: AAF1-GFP-natMX4/aaf1:: AAF1-GFP-natMX4*
rsf2Δ	*MATα/a, rsf2::hphMX4/rsf2::hphMX4*
aaf1Δrsf2Δ	*MATα/a, aaf1::kanMX4/aaf1::kanMX4, rsf2::hphMX4/rsf2::hphMX4*
ald4Δ	*MATα/a, ald4::hphMX4/ald4::hphMX4*
ald4Δaaf1Δ	*MATα/a, ald4::hphMX4/ald4::hphMX4, aaf1::kanMX4/aaf1::kanMX4*
ald4Δrsf2Δ	*MATα/a, ald4::kanMX4/ald4::kanMX4, rsf2::hphMX4/rsf2::hphMX4*
ald4Δaaf1Δrsf2Δ	*MATα/a, ald4::natMX4/ald4::natMX4, aaf1::kanMX4/aaf1::kanMX4, rsf2::hphMX4/rsf2::hphMX4*
ald6Δ	*MATα/a, ald6::hphMX4/ald6::hphMX4*
ald6Δaaf1Δ	*MATα/a, ald6::hphMX4/ald6::hphMX4, aaf1::kanMX4/aaf1::kanMX4*
ald6Δrsf2Δ	*MATα/a, ald6::kanMX4/ald6::kanMX4, rsf2::hphMX4/rsf2::hphMX4*
ald6Δaaf1Δrsf2Δ	*MATα/a, ald6::natMX4/ald6::natMX4, aaf1::kanMX4/aaf1::kanMX4, rsf2::hphMX4/rsf2::hphMX4*
ald4Δald6Δ	*MATα/a, ald4::hphMX4/ald4::hphMX4, ald6::natMX4/ald6::natMX4*
ald4Δald6Δaaf1Δ	*MATα/a, ald4::hphMX4/ald4::hphMX4, ald6::kanMX4/ald6::kanMX4, aaf1::natMX4/aaf1::natMX4*
ald4Δald6Δrsf2Δ	*MATα/a, ald4::kanMX4/ald4::kanMX4, ald6::natMX4/ald6::natMX4, rsf2::hphMX4/rsf2::hphMX4*
WT/*RSF2*↑	*MATα/a, kanMX4-pPGK1-RSF2/kanMX4-pPGK1-RSF2*
WT/*AAF1*↑	*MATα/a, kanMX4-pPGK1-AAF1/kanMX4-pPGK1-AAF1*
ald4Δ/RSF2↑	*MATα/a, ald4::hphMX4/ald4::hphMX4, kanMX4-pPGK1-RSF2/kanMX4-pPGK1-RSF2*
ald4Δ/AAF1↑	*MATα/a, ald4::hphMX4/ald4::hphMX4, kanMX4-pPGK1-AAF1/kanMX4-pPGK1-AAF1*
ald6Δ/RSF2↑	*MATα/a, ald6::hphMX4/ald6::hphMX4, kanMX4-pPGK1-RSF2/kanMX4-pPGK1-RSF2*
ald6Δ/AAF1↑	*MATα/a, ald6::hphMX4/ald6::hphMX4, kanMX4-pPGK1-AAF1/kanMX4-pPGK1-AAF1*
ald4Δald6Δ/RSF2↑	*MATα/a, ald4::hphMX4/ald4::hphMX4, ald6::natMX4/ald6::natMX4, kanMX4-pPGK1-RSF2/kanMX4-pPGK1-RSF2*
ald4Δald6Δ/AAF1↑	*MATα/a, ald4::hphMX4/ald4::hphMX4, ald6::natMX4/ald6::natMX4, kanMX4-pPGK1-AAF1/kanMX4-pPGK1-AAF1*
AAF1-GFP/*NIC96*-mCherry	*MATα/a, AAF1-GFP-natMX4/AAF1-GFP-natMX4, NIC96-mCherry-hphMX4/NIC96-mCherry-hphMX4*

used commercial wine yeast strain that is a homozygous diploid strain (Bradbury *et al.*, 2006; Deed *et al.*, 2011). Rich medium YPD (DIFCO, 1% yeast extract, 2% peptone, 2% dextrose) was used for routine culture. For antibiotic selection, G418/geneticin (200 μg mL^{-1}), hygromycin B (200 μg mL^{-1}), or cloNAT (100 μg mL^{-1}) was added to YPD agar plates. The plates for spot assays were YPD agar and YPD agar plus 0.1% of potassium acetate. Fermentations were performed in triplicate in filter-sterilized 2009 Chardonnay grape juice obtained from Calona Vineyards (Okanagan, BC, Canada) at 20 and 14°C, as previously described (Luo & van Vuuren, 2008). Fermentation progress in each flask was monitored by weight loss, which reflects CO_2 release.

Strain construction

For the construction of null mutants in the M2 yeast strain, the entire ORF of the target gene was replaced by homologous recombination with antibiotic resistance genes. The geneticin-resistance gene, *kanMX4,* was amplified from the plasmid pUG6 (Guldener *et al.*, 1996), hygromycin-resistance gene *hphMX4* from pAG32 (Goldstein & McCusker, 1999), and cloNAT-resistance gene *natMX4* from pAG25 (Goldstein & McCusker, 1999). The PCR primers contained 15–19 nucleotides at the 3′ end designed to amplify the cassette, and 45–70 gene-specific nucleotides at the 5′ ends to target the genes. High fidelity iProof kits (Bio-Rad) were used for PCR amplification.

PCR products were transformed into the M2 strain by the standard lithium acetate method. Transformants were selected on YPD plates containing antibiotics, and gene deletion was confirmed by colony PCR. The heterozygous transformants were sporulated, dissected, and selected by antibiotics. Because M2 is homothallic, the resultant meiotic haploid deletion mutants can switch mating type and mate with each other to form homozygous diploid strains. The correct replacement and integration on both chromosomes in the diploid strains were confirmed by colony PCR.

For C-terminal GFP tagging of Aaf1p, the GFP-*NatMX4* was amplified from the pGFP+NAT plasmid (Vizeacoumar *et al.*, 2006). For C-terminal mCherry tagging of the nuclear membrane marker Nic96p, the mCherry-*HphMX4* cassette was amplified from the pKT-mCherry-*HphMX4* plasmid (Sheff & Thorn, 2004).

For promoter replacement of *AAF1* and *RSF2*, the fragment containing the marker gene *kanMX4* and the 788-bp *PGK1* promoter sequence was amplified from the plasmid pCW1 (Walkey *et al.*, 2012). Oligos used in this study are listed in Table 2.

Wine analysis

Wine samples were periodically removed from the flasks without introducing air, and measured for the levels of glucose, fructose, glycerol, acetic acid, and ethanol by HPLC (Adams & van Vuuren, 2010). Acetaldehyde was measured using an acetaldehyde assay kit (Megazyme). All assays were conducted in triplicate.

Microscopy

The M2 yeast strain carrying a GFP tag at the C-terminus of Aaf1p and an mCherry tag at the C-terminus of Nic96p was imaged after fermentation of Chardonnay grape must for 7 days. An aliquot of yeast cells was immobilized under an agarose gel slab, and immediately visualized and photographed by fluorescence microscopy with a Zeiss Axio Observer Z.1 microscope. The microscopic images were processed with Gauss binomial smoothing (kernel size = 3), and an unsharp mask was applied (radius = 2, strength = 1).

Results

The *S. cerevisiae aaf1Δ* mutant produces less acetic acid in wine

To confirm whether *AAF1* regulates acetic acid production in wine, we fermented the Chardonnay grape must with the wild-type M2 and the *aaf1Δ* strains to completion and monitored cell growth, sugar depletion, ethanol production, and glycerol and acetic acid levels (Fig. 1). The *aaf1Δ* strain behaved very similarly to the wild-type strain with respect to growth rate (Fig. 1a), fructose and glucose depletion (Fig. 1b and c), and glycerol and ethanol production (Fig. 1d and f) during fermentation. However, the production of acetic acid in the *aaf1Δ* strain was significantly less than in the wild-type strain even only after 4 days of fermentation (Fig. 1e). After completion of fermentation, the acetic acid levels in the *aaf1Δ* strain were only 67.8% of that in the wild-type strain (Fig. 1e). To further confirm that the reduction of acetic acid in the deletion mutant is indeed caused by the absence of the *AAF1* gene, we introduced a tagged version of *AAF1* (*AAF1-GFP*) into the deleted *AAF1* locus under the control of the endogenous *AAF1* promoter. We found that *AAF1-GFP* restored the acetic acid content to wild-type levels (Fig. 1e) and had no effect on the other fermentation parameters (Fig. 1a–d and f). These results confirmed that the reduction of acetic acid levels in the *aaf1Δ* strain was due solely to the absence of Aaf1p. As well, these results showed that the C-terminal GFP-tagged version of Aaf1p was fully functional.

Table 2. Oligonucleotides used in this study

Target ORF/gene name	Forward primer	Reverse primer	Purpose
n/a	GACTGTCACTGATCGTACATGC	CCAAGTCTGACTATCGTAGTGC	Common primers for re-amplification of deletion cassette
KanMX4	AATGCTGGTCGCTATACTGC	CTGCAGCGAGGAGCCGTAAT	Null mutant check
AAF1	CCCAGTTGTCTTCTGTTCTATCAG CAGCGAATATTTCAGCTTCTTGT AATTGTACGTTGCATCTGCCATG agctgaagcttcgtacg	TTTTTCAATTTGCCCTAAAGAAC TAATATAATGTTACATACGGAT ATGCTAAATATCTATCTAAAGT CTAtacgactcactataggg	Deletion cassette amplification
AAF1	TGTTACCAATCAAGCGCTGG	ATAGACGATAGCACTTTGGG	Null mutant check
RSF2	GGTAACTACGCGAGCAACTTCTA TTAAGAGAAATAATTTTTGGGAA ATGGCCTGTTTCGGagctgaagcttc gtacgc	TCTAAGCTTTAATTCTGTAAATA CTATAGTATAGAGACGGCCGC CATTATATATTTGTAAtaggccact agtggatctg	Deletion cassette amplification
RSF2	ATTTCTTAAGATGGCAGGAC	TGCTTTCTTCATTGTCATCG	Null mutant check
ALD4	AGGATTAGAAGTATCTGGAAAAC CAACCAAGAAAACTACAATAAC AAAAATAAATAAAGCagctgaagct tcgtacgc	GACAGAATATTTAATTTTATGTA TGTAAGCATCGATTGGACACCA GGCTTATTGATGACCtaggccact agtggatctg	Deletion cassette amplification
ALD4	AGCCAACTGTCTTTGGTGAC	AAGTTTCATCAAGGTCTCTG	Colony PCR
ALD6	TAGAAGAAAAAACATCAAGAAAC ATCTTTAACATACACAAACACAT ACTATCAGAATACAagctgaagcttc gtacgc	GTAAGACCAAGTAAGTTTATAT GAAAGTATTTTGTGTATATGAC GGAAAGAAATGCAGGTtaggcc actagtggatctg	Deletion cassette amplification
ALD6	ACCAACCGTTTTCTACGATG	TAGCAGTTGTTGTACACTAG	Null mutant check
p*PGK1-AAF1*	GTCTTCTGTTCTATCAGCAGCGAA TATTTCAGCTTCTTGTAATTGTAC GTTGCATCTGCCttaatacgactcact ataggg	AGCATAGATAGTGGAGGATATA TCTCGTTTGATCGGAAGTCCTT TGAATTCTTCCGATGAcattgtttt atatttgttgtaaaaagtag	Amplification of kanMX4-p*PGK*1
AAF1	AAAGGGTTTCTCGTTCGTATGTGC	TCAAACAGAATTGTCCGAATCG	Overexpression cassette check
p*PGK1-RSF2*	GGTAACTACGCGAGCAACTTCTA TTAAGAGAAATAATTTTTGGGAA ATGGCCTGTTTCGGttaatacgactc actataggg	TCGAGCGGCCGCGGTTAGTATG CATAATGCAGGCGCCCCTCGTC CAAATGCGAACGGTTCcattgtttt atatttgttgtaaaaagtag	Amplification of kanMX4-p*PGK1*
RSF2	GTGAACCACTCCAGGGGC	GAAAAGTGCCCAGCAACACG	Overexpression cassette check
NIC96-mCherry	ATTCAATACAGAATGCCAAGGGA AACGTACAGCACTTTAATTAATA TAGACGTCTCTCTAggtgacggtgct ggttta	CTAAGTATGCGCGCATACTGATA TATAGATATAAACAAAAATATA CAATATTTAAAAAAAtcgatgaat tcgagctcg	Amplification of mCherry-hphMX4
NIC96-mCherry	TTTGTTGATTATCACTCTAAGCTG TATATCG	CCCCAATGCTTATGAAATCCAAC C	mCherry fusion check
AAF1-GFP	CATGCCTTACAATCTAGGGCTATT TACAATATCAACCACAGGAAATC TGTAAACAGTGTAGGTgaagctca aaaacttaat	ATTTGCCCTAAAGAACTAATATA ATGTTACATACGGATATGCTAA ATATCTATCTAAAGTGCTgacggt atcgataagctt	Amplification of GFP-natMX4
GFP	TCACATGATGTTACCAATCAA GCG	ATAGACGATAGCACTTTGGG	GFP fusion check

Aaf1p is localized in the nucleus under wine fermentation conditions

Aaf1p contains a zinc finger at its N-terminus and is predicted to function as a transcription factor. A high-throughput experiment demonstrated that Aaf1p is a nuclear protein under standard laboratory growth conditions (Huh *et al.*, 2003). Therefore, we tested Aaf1p localization under wine fermentation conditions. The M2 strain carrying Aaf1p-GFP and Nic96p-mCherry (a nuclear membrane marker) was used to ferment Chardonnay grape must for 7 days, at which point the yeast cells were examined by fluorescence microscopy. As shown in Fig. 2, the majority of the Aaf1p-GFP signal was detected in the nucleus, which was surrounded by the Nic96p-mCherry nuclear membrane protein.

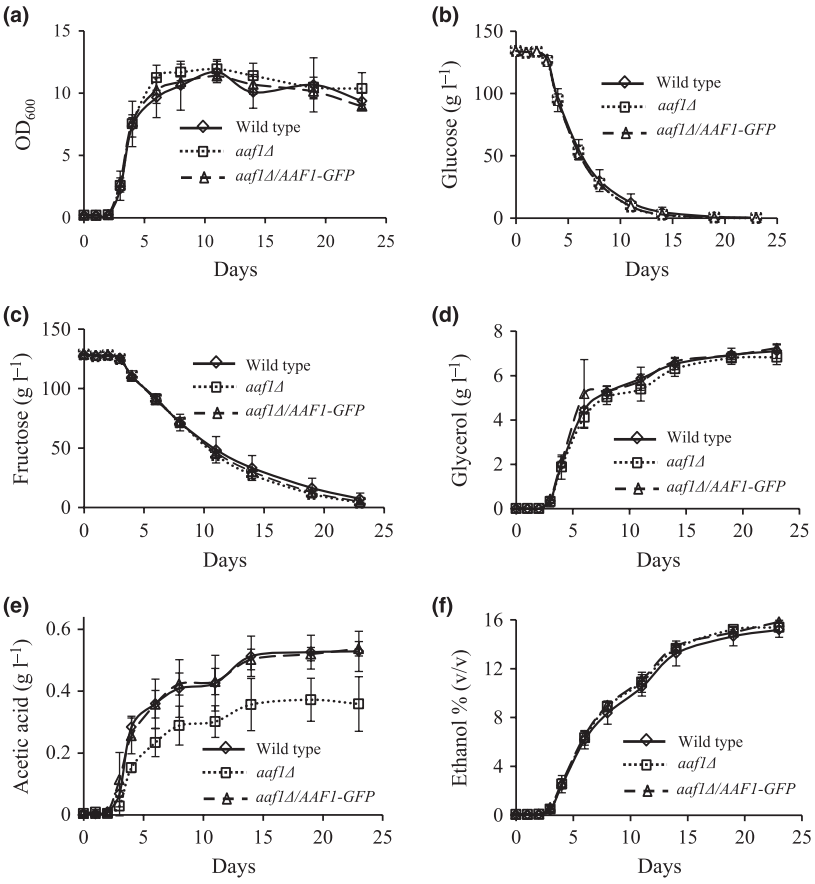

Fig. 1. Cell density and glucose, fructose, glycerol, acetic acid, and ethanol concentrations during the Chardonnay grape must fermented by the wild-type M2, *aaf1Δ*, and *aaf1Δ/AAF1-GFP* strains. Yeast cells were inoculated into Chardonnay grape must, and fermented at 20°C to completion. Fermentations were conducted in triplicate. At the indicated time points, aliquots were withdrawn, and the yeast cell density was assayed by the standard OD_{600} method. Glucose, fructose, glycerol, acetic acid and ethanol were assayed by HPLC (Adams & van Vuuren, 2010). Each data point represents the mean from three separate fermentations. Error bars represent standard deviations.

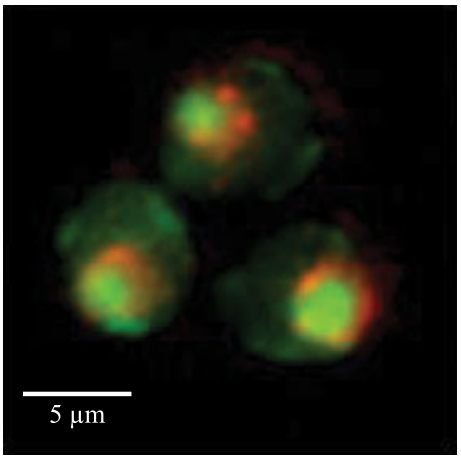

Fig. 2. Aaf1p is localized in the yeast nucleus. An M2 strain carrying a GFP tag at the C-terminus of Aaf1p and an mCherry tag at the C-terminus of Nic96p was inoculated into Chardonnay grape must. An aliquot of yeast cells was removed after 7 days at 20°C, immobilized under an agarose gel slab, and immediately visualized and photographed by fluorescence microscopy with a Zeiss Axio Observer Z.1 microscope. The green color represents Aaf1p-GFP, and the red color represents Nic96p-mCherry.

Deletion of *AAF1* is detrimental to *ald6Δ* and *ald4Δald6Δ* mutants

We have demonstrated Aaf1p regulates the expression of the *ALD4* and *ALD6* genes in standard laboratory growth conditions (Walkey *et al.*, 2012). Therefore, we tested whether *AAF1* has a synergistic effect on the acetic acid production with its target genes *ALD4* and *ALD6*, as well as with its paralog *RSF2*. Single, double, or triple deletions of *AAF1*, *RSF2*, *ALD4*, and *ALD6* were constructed in the industrial *S. cerevisiae* M2 strain. Some of the mutant strains grew slowly in YPD, but recovered to wild-type level when grown in YPD plus acetate. To compare the fitness of these deletion mutants, we cultured mutant and wild-type strains to stationary phase in YPD plus 0.1% potassium acetate and then spotted serial dilutions of the same amount of cells on YPD agar and YPD agar plus 0.1% potassium acetate (Fig. 3). The *aaf1Δ* strain grew slightly slower than the wild-type M2 strain in YPD media. Synthetic sickness was observed between *aaf1Δ* and *ald6Δ* and between *ald4Δ* and *ald6Δ* in YPD, but not between *aaf1Δ* and *ald4Δ*, suggesting that Aaf1p is primarily responsible for transcriptional activation of

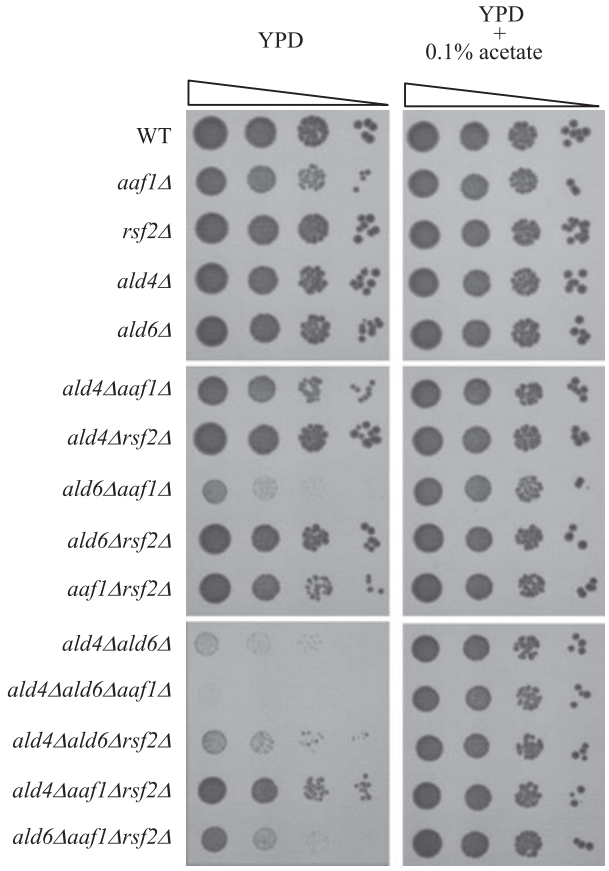

Fig. 3. Deletion of *AAF1* is detrimental to *ald6Δ* and *ald4Δald6Δ* mutants. Cells were grown in YPD broth plus 0.1% potassium acetate to stationary phase and diluted to OD_{600} = 0.5; 3 μL of cells were spotted onto YPD, and YPD plus 0.1% potassium acetate plates with a 10 times serial dilution, and incubated at 30°C for 2 days.

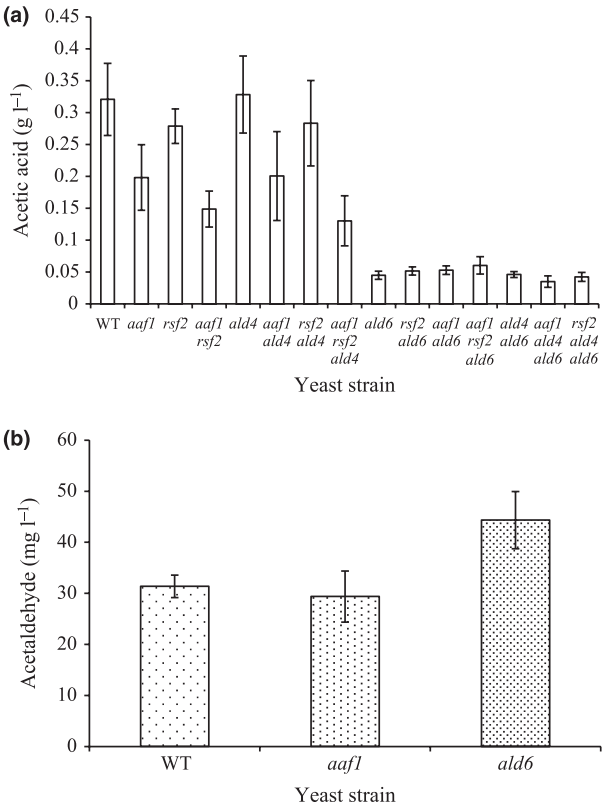

Fig. 4. Effects of deletion of the *AAF1*, *RSF2*, *ALD4,* and *ALD6* genes on the levels of (a) acetic acid and (b) acetaldehyde in Chardonnay wine. Fermentations were conducted in triplicate to completion at 20°C. Each data point represents the mean from three separate fermentations. Error bars represent standard deviations.

ALD4 and that there is still enough Ald6p expressed in *aaf1Δald4Δ* strain for viability. The absence of *AAF1* exacerbated the poor growth of the *ald4Δald6Δ* in YPD, indicating that Aaf1p may regulate the expression of other gene(s), which contribute to the growth of *ald4Δald6Δ* mutant. Importantly, all growth defects were rescued by the addition of potassium acetate to the media, highlighting the roles of Aaf1p, Ald4p, and Ald6p in the cellular acetate biosynthesis. Absence of *RSF2* had no obvious effect on the growth of *aaf1Δ*, *ald4Δ*, *ald6Δ*, and *ald4Δald6Δ* mutants, indicating that Rsf2p functions differently from its paralog Aaf1p.

AAF1, *RSF2*, *ALD4,* and *ALD6* do not have synthetic or synergistic roles in regulating acetic acid production in wine

We then fermented Chardonnay grape juice at 20°C with all of the deletion mutants previously described to test their effect on the acetic acid levels in wine. The weight loss during the course of fermentation showed that the *aaf1Δald6Δ*, *ald4Δald6Δ*, *rsf2Δald4Δald6Δ*, *aaf1Δald4Δald6Δ*, and *rsf2Δaaf1Δald6Δ* mutant strains, which grew poorly on YPD, fermented the grape juice much more slowly than the wild-type strain and the other mutants that did not have growth defects on YPD. After 20 days of fermentation, 4–7% (w/v) of sugar remained in the grape must fermented by *aaf1Δald6Δ*, *ald4Δald6Δ*, *rsf2Δald4Δald6Δ*, *aaf1Δald4Δald6Δ*, and *rsf2Δaaf1Δald6Δ* strains; sugar was almost completely consumed by the wild-type and the other deletion mutant strains. It took another 20 days for the sluggish deletion mutant strains to complete their fermentations. The levels of acetic acid in final wines are shown in Fig. 4a. As expected, cells lacking Aaf1p produced 39.2% less acetic acid than the wild-type M2 yeast. Deletion of *ALD6* reduced the acetic acid levels by 86%. Deletion of *RSF2* and *ALD4* had no significant effect on acetic acid production. Double or triple deletion mutants did not significantly affect the acetic acid levels compared with the single mutants, indicating that these genes do

not have synthetic or synergetic roles in regulating acetic acid production in wine. For example, the *aaf1Δ* mutant and the *aaf1Δald4Δ* mutant had similar acetic acid levels, which corroborate the spot assay data that the double mutant does not have an additional growth defect. Although the *ald6Δaaf1Δ* double mutant has a synthetic growth defect, we did not observe a reduction in acetic acid production in the *ald6Δaaf1Δ* mutant compared with the *ald6Δ* mutant alone (Fig. 4a). This is likely due to the fact that acetic acid levels are already extremely low in the *ald6Δ* mutant.

Absence of Aaf1p does not increase acetaldehyde levels in wine

In yeast, acetate is synthesized by oxidation of acetaldehyde, which is catalyzed by acetaldehyde dehydrogenase. The decrease of acetic acid production in wine fermented by the *ald6Δ* and *aaf1Δ* deletion strains could result in acetaldehyde accumulation. Acetaldehyde, at low levels, imparts a pleasant fruity aroma to wine, but at higher concentrations, turns it into a pungent irritating odor (Liu & Pilone, 2000; Styger *et al.*, 2011). Therefore, we assayed acetaldehyde content in the final wine fermented with *ald6Δ*, *aaf1Δ,* and wild-type M2 strains. As shown in Fig. 4b, acetaldehyde in wine produced with the *ald6Δ* mutant increased 41.4% compared with that in wild-type strain, suggesting that the *ald6Δ* strain is not suitable for industrial production of wine. In contrast, no significant changes were observed in acetaldehyde content during the *aaf1Δ* fermentation when compared with wine produced with the WT yeast strain. We further conducted fermentations at 14°C with the *aaf1Δ* mutant and wild-type M2 strains to avoid acetaldehyde evaporation because its' boiling temperature is only 19°C. As shown in Table 3, the absence of Aaf1p reduced the acetic acid concentration by 38.3% compared with the wild-type strain, but did not significantly affect acetaldehyde levels in the wine fermented at 14°C. These results are encouraging for the commercialization of *AAF1* deletion yeast strains to minimize VA in wine.

Table 3. The *Saccharomyces cerevisiae aaf1Δ* mutant produces significantly less acetic acid in the final wine without increasing acetaldehyde levels

Yeast strain	Glycerol (g L^{-1})	Acetic acid (g L^{-1})	Acetaldehyde (mg L^{-1})	Ethanol % (v/v)
WT	4.69 ± 0.24	0.51 ± 0.07	36.72 ± 1.27	13.79 ± 0.17
aaf1Δ	4.47 ± 0.03	0.31 ± 0.02	35.84 ± 3.36	13.71 ± 0.07

Fermentations were conducted in triplicate in Chardonnay grape must for 64 days at 14°C to completion.

Overexpression of *AAF1* significantly increases acetic acid production in wine in an Ald6p-dependent manner

We further tested the effects of overexpression of *AAF1* and *RSF2* in wild-type, *ald4Δ, ald6Δ,* and *ald4Δald6Δ* strains on acetic acid and acetaldehyde levels in Chardonnay grape must fermented to completion. Compared with the wild-type strain, the acetic acid levels were increased 5.5-fold when Aaf1p was overexpressed in wild-type (WT/*AAF1*↑) and *ald4Δ (ald4Δ/AAF1*↑) strains, but decreased 65.6% and 62.8% in *ald6Δ* (*ald6Δ/AAF1*↑) and *ald4Δald6Δ* (*ald4Δald6Δ/AAF1*↑) strains, respectively (Fig. 5a). No effect was detected in *RSF2* overexpression strains. These findings suggest that Aaf1p regulates the acetic acid levels mainly in an Ald6p-dependent manner, and *RSF2* has little effect on acetic acid production in wine. When compared with their deletion counterparts,

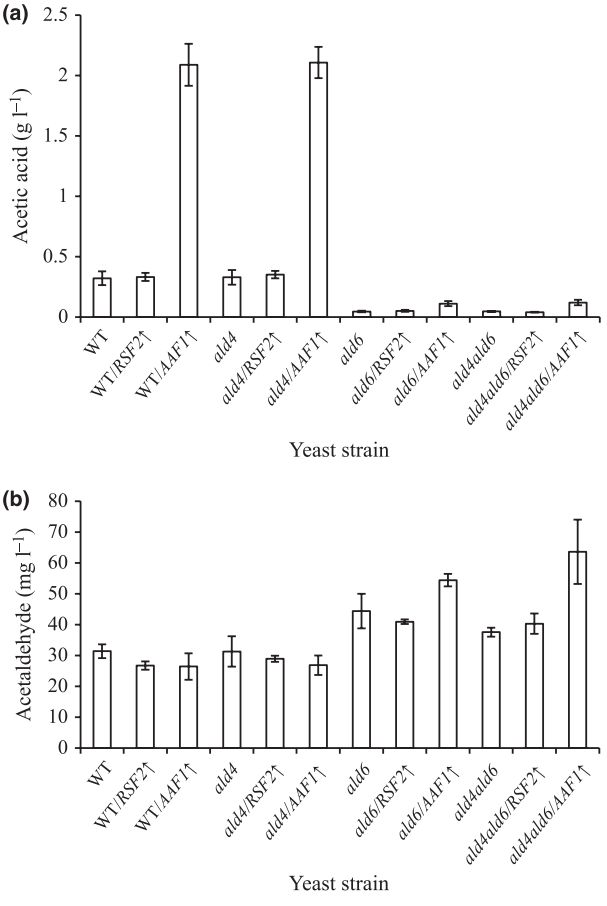

Fig. 5. Effects of overexpression of *AAF1* and *RSF2* in *ALD4* and *ALD6* deletion mutants on the levels of (a) acetic acid and (b) acetaldehyde in Chardonnay wine. Fermentations were conducted in triplicate to completion at 20°C. Each data point represents the mean from three separate fermentations. Error bars represent standard deviations. '↑' indicates gene overexpression.

the *ald6Δ/AAF1*↑ and *ald4Δald6Δ/AAF1*↑ strains produced 1.46 and 1.59 times more acetic acid, as well as 22.6% and 69.4% more acetaldehyde in wine, respectively (Fig. 5b and Table 4). These results suggest that Aaf1p may also regulate acetic acid production independently of Ald4p and Ald6p.

Discussion

Recent advances in high-throughput genomic technologies make it possible to develop molecular profiles that lead to hypotheses regarding gene function(s). Ideally, the insights gained from these high-throughput techniques will help answer many fundamental questions in biology. We analyzed the transcriptome of an industrial wine yeast strain (Vin 13) throughout a wine fermentation, and discovered 62 nonannotated FSR genes whose expression was highly induced during fermentation (Marks *et al.*, 2008); these FSR genes may play important roles during grape must fermentation. In the present study, we found that deletion of the FSR gene, *AAF1,* reduced acetic acid in wine by 39.2% at 20°C compared with the WT M2 yeast strain. Further experiments confirmed that the protein encoded by this gene, a zinc-finger transcription factor, is indeed localized in the nucleus under wine fermentation conditions, and it regulates acetic acid levels in wine mainly via Ald6p, a cytosolic aldehyde dehydrogenase that catalyzes the oxidation of acetaldehyde to acetate.

The yeast metabolic pathways contributing to acetic acid formation in wine have not yet been completely elucidated (Boulton *et al.*, 1996; Vilela-Moura *et al.*, 2011). It has been proposed that acetic acid in wine is mainly produced through the pyruvate dehydrogenase (PDH) bypass pathway (Vilela-Moura *et al.*, 2011). We showed that strains lacking Ald6p, a major enzyme in the PDH bypass pathway, produced an 86% reduction in acetic acid levels in Chardonnay wine, while deletion of *ALD4* had no significant effect. These results are in agreement with previous studies in laboratory media and synthetic must (Remize *et al.*, 2000; Saint-Prix *et al.*, 2004; Walkey *et al.*, 2012). Although deletion of *ALD4* had no significant effect on the acetic acid production during fermentation, it delayed growth in *ald6Δ* mutants in YPD (Fig. 2, Wang *et al.*, 1998; Remize *et al.*, 2000). One explanation for this synthetic sickness between *ald4Δ* and *ald6Δ* is that *ALD6* deletion induced *ALD4* expression and thus compensated for the lack of Ald6p (Saint-Prix *et al.*, 2004). Our results of synthetic sickness between *aaf1Δ* and *ald6Δ*, but not between *aaf1Δ* and *ald4Δ*, suggested that Aaf1p was primarily responsible for transcriptional activation of *ALD4.*

From the comparison of the acetic acid levels produced by *aaf1Δ* (−39.2%) and *ald6Δ* (−86%), it seems that a significant portion of Ald6p activity is regulated independently of Aaf1p. Therefore, other gene(s) are likely involved in regulating Ald6p activity. Identification of these gene(s) could be helpful to control acetic acid production in wine. *RSF2*, the paralog of *AAF1*, has been reported to regulate the levels of *ALD6* expression (Grabowska & Chelstowska, 2003); however, we did not observe that deletion of *RSF2* had a significant effect on the acetic acid production both in laboratory media (Walkey *et al.*, 2012) and during wine fermentation (Fig. 5a). As well, the synthetic sickness of the *ald4Δald6Δ* double mutant was not regenerated with an *ald4Δrsf2Δaaf1Δ* strain, suggesting that other transcription factor(s) may still be able to maintain *ALD6* expression in the absence of *RSF2* and *AAF1*. Yap1p, a member of the AP-1 family of transcription factors, has been reported to directly bind the promoters of *ALD5* and *ALD6* genes in a ChIP-chip genome-wide location analysis (Salin *et al.*, 2008). Furthermore, wine yeast with mutations in *YAP1* produced less acetic acid during fermentation and showed lower ALD activity (Cordente *et al.*, 2013), suggesting that Yap1p might regulate the expression of *ALD* genes.

The dramatic reduction of acetic acid levels by deletion of *ALD6* was accompanied by significantly increased acetaldehyde levels (Fig. 4b). This phenomenon limits the industrial application of the *ald6Δ* strain, as high levels of acetaldehyde are deleterious to wine quality (Styger *et al.*, 2011). On the other hand, the *aaf1Δ* strain produced substantially less acetic acid in wine, but did not increase the levels of acetaldehyde (Fig. 4b and Table 3); ethanol and glycerol levels were unaffected (Fig. 1). Therefore, inactivation of Aaf1p by substitution of single or a few nucleotides in the coding sequences by breeding or recombinant methods seems to be promising to improve industrial yeast strains for the production of wine with low volatile acidity.

Table 4. Overexpression of *AAF1* in *ald6Δ* and *ald4Δald6Δ* deletion strains increased the acetic acid and acetaldehyde levels in wine

	Acetic acid		Acetaldehyde	
Strain	Concentration ($g\ L^{-1}$)	Relative change	Concentration ($mg\ L^{-1}$)	Relative change
ald6Δ	0.045 ± 0.006	1	44.346 ± 5.607	1
ald6Δ/RSF2↑	0.050 ± 0.008	1.124	40.919 ± 0.729	0.923
ald6Δ/AAF1↑	0.110 ± 0.020	2.460	54.381 ± 2.025	1.226
ald4Δald6Δ	0.046 ± 0.005	1	37.537 ± 1.452	1
ald4Δald6Δ/RSF2↑	0.040 ± 0.003	0.868	40.295 ± 3.299	1.073
ald4Δald6Δ/AAF1↑	0.119 ± 0.020	2.594	63.593 ± 10.42	1.694

'↑' indicates gene overexpression. Relative change indicates ratio between overexpression strains and their deletion counterparts.

An alternative pathway for acetate production in *S. cerevisiae* has been suggested (Saint-Prix *et al.*, 2004). Our results, the genetic interaction between *AAF1*, *ALD4,* and *ALD6* (Fig. 3), and the overexpression of *AAF1* in *ald6Δ* and *ald4Δald6Δ* mutants (Fig. 5a), seem to support this conclusion. Firstly, deletion of *AAF1* exacerbated the growth defect of *ald4Δald6Δ* mutants in YPD (Fig. 3). Secondly, overexpression of *AAF1* in *ald6Δ* and *ald4-Δald6Δ* mutants produced more than a twofold increase in acetic acid in wine compared with acetic acid production in the *ald6Δ* and *ald4Δald6Δ* strains (Table 4). These results suggest that the transcription factor Aaf1p may regulate other genes involved in the production of acetic acid. However, acetaldehyde levels were also increased by overexpression of *AAF1* in *ald6Δ* and *ald4Δald6Δ* (Fig. 5b), indicating that these other genes could be involved upstream of acetaldehyde dehydrogenase in the PDH bypass, or otherwise be indirectly involved in acetate biosynthesis. Investigations are underway to identify other potential Aaf1p target genes.

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Authors' contribution

Z.L. and C.J.W. contributed equally to this work.

Patent

A patent application on low VA producing wine yeasts has been submitted to the US Patent and Trade Mark Office (13-011 USP).

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