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Molecular characterization of the *Saccharomycopsis fibuligera ATF* genes, encoding alcohol acetyltransferase for volatile acetate ester formation[§]

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Aroma ester components produced by fermenting yeast cells via alcohol acetyltransferase (AATase)-catalyzed intracellular reactions are responsible for the fruity character of fermented alcoholic beverages, such as beer and wine. Acetate esters are reportedly produced at relatively high concentrations by non-*Saccharomyces* species. Here, we identified 12 *ATF* orthologues (*SfATFs*) encoding putative AATases, in the diploid genome of *Saccharomycopsis fibuligera* KJJ81, an isolate from wheat-based Nuruk in Korea. The identified SfATF proteins (SfAtfp) display low sequence identities with *S. cerevisiae* Atf1p (between 13.3 and 27.0%). All SfAtfp identified, except SfAtf(A)4p and SfAtf(B)4p, contained the activation domain (HXXXD) conserved in other Atf proteins. Culture supernatant analysis using headspace gas chromatography mass spectrometry confirmed that the recombinant *S. cerevisiae* strains expressing SfAtf(A)2p, SfAtf(B)2p, and SfAtf(B)6p produced high levels of isoamyl and phenethyl acetates. The volatile aroma profiles generated by the SfAtf proteins were distinctive from that of *S. cerevisiae* Atf1p, implying difference in the substrate preference. Cellular localization analysis using GFP fusion revealed the localization of SfAtf proteins proximal to the lipid particles, consistent with the presence of amphipathic helices at their N- and C-termini. This is the first report that systematically characterizes the *S. fibuligera ATF* genes encoding functional AATases responsible for acetate ester formation using higher alcohols as substrate, demonstrating their biotechnological potential for volatile ester production.

Keywords: *Saccharomycopsis fibuligera*, volatile flavor, acetate esters, alcohol-*O*-acetyltransferases

Introduction

Microbial activity during fermentation for alcoholic beverage, such as beer and wine, is considerably more complex than merely producing ethanol, usually involving the activity of diverse microorganisms, which affect the aroma and flavor of fermented beverages. Volatile esters are secondary metabolites produced by yeast and fungi during fermentation, and there are two major aroma-esters in fermented beverages: the acetate esters and the medium-chain fatty acid (MCFA) ethyl esters (Saerens *et al*., 2010; Hu *et al*., 2018). Acetate esters are produced from acetic acid (acetate) with ethanol or a higher alcohol (fusel alcohol) through the action of alcohol acetyltransferase (AATases; EC 2.3.1.84) (Yoshioka and Hashimoto, 1981). The acetate esters from higher alcohols are derived from branched-chain amino acids (leucine, valine, and isoleucine), aromatic amino acids (phenylalanine, tyrosine, and tryptophan), and the sulfur-containing amino acid methionine, which are converted to α-keto acids via transamination (Hazelwood *et al*., 2008). Subsequently, the α-keto acids are decarboxylated to an aldehyde by a 2-keto acid decarboxylase, and ultimately either reduced to a higher alcohol by an alcohol dehydrogenase/aldehyde reductase or oxidized to an acid by an aldehyde dehydrogenase. The fusel alcohols and acetyl coenzyme A (acetyl-CoA), which are produced from pyruvate or acetic acid, can be condensed via the action of alcohol-*O*-acetyltransferases (Atfs). In the traditional yeast *Saccharomyces cerevisiae*, there are two Atf enzymes (Atf1p and Atf2p) catalyzing these reactions (Fujii *et al*., 1994; Nagasawa *et al*., 1998). The second group of aroma-esters, MCFA-ethyl ester is produced in *S. cerevisiae* through the condensation of the acyl chain from fatty acid-coenzyme A and ethanol by Eeb1p and Eht1p, which encode acetyl-CoA:ethanol *O*-acyltransferase (Saerens *et al*., 2006). MCFA-ethyl ester includes aniseed or apple aroma ethyl hexanoate and sour apple scent ethyl octanoate (Saerens *et al*., 2010).

The most important flavor acetate esters in fermented foods and beverages are the ethyl, isobutyl, isoamyl, and phenethyl acetates that provide the scent of fruity, sweet, banana, and rose, respectively, during fermentation (Saerens *et al*., 2010). The alcohol-*O*-acetyltransferases are key enzymes for the production of acetate esters by transferring the acetyl chain from an acetyl-coenzyme A to a fusel alcohol derived from amino acids (Tashiro *et al*., 2015b). The knockout of the *ATF1* and *ATF2* genes, encoding Atf1p and Atf2p, in *S. cerevisiae* showed

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significantly reduced levels of a broad range of acetate esters, while *ATF1* and *ATF2* overexpressing strains exhibited an increased production of numerous acetate esters (Lilly *et al*., 2000, 2006; Verstrepen *et al*., 2003). Of these two AATase proteins, Atf1p plays the main role in producing acetate esters (Verstrepen *et al*., 2003), whereas Atf2p is important for sterol detoxification by facilitating secretion of acetylated overload-sterols into the culture medium (Tiwari *et al*., 2007). A previous study showed that the Atf1p and Atf2p in *S. cerevisiae* localize to the lipid droplets (LDs) via the endoplasmic reticulum (ER) in the stationary phase (Verstrepen *et al*., 2004). The N- and C-terminal dual amphipathic helices structure of *S. cerevisiae* Atf proteins was shown to be important for ER and LD association (Lin and Wheeldon, 2014). Orthologues of these two enzymes were discovered and characterized in other yeasts, such as *Kluyveromyces lactis* (Van Laere *et al*., 2008), three *Saccharomyces sensu stricto* species namely *S. pastorianus* (Yoshimoto *et al*., 1998, 1999), *S. kudriavzevii*, and *S. uvarum* (Stribny *et al*., 2016), and two *Wickerhamomyces* species including *W. anomalus* (*Pichia anomala*) (Schneider *et al*., 2012; Kruis *et al*., 2017) and *W. ciferrii* (*P. ciferrii*) (ter Veld *et al*., 2013). Interestingly, the ER and LD associations were observed in all AATases from *Saccharomyces* yeast, exhibiting the N- and C-amphipathic helices, but not in those from *K. lactis* and *W. anomalus*, which lacked terminal amphipathic helices (Lin and Wheeldon, 2014).

The amylolytic yeast *Saccharomycopsis fibuligera* (synonymous with *Endomyces fibuligera*) is an industrial strain with a long history of use in various biotechnological applications and industrial fermentation (Chi *et al*., 2009). *Saccharomycopsis fibuligera* is found worldwide as the major amylolytic yeast utilized in indigenous food fermentation using rice and commonly found as a dominant yeast species in traditional Asian alcoholic starters for rice wine production. A previous study investigating the characteristics of yeasts isolated from traditionally fermented *masau* (*Ziziphus mauritiana*) fruits identified *S. fibuligera* as the responsible yeast strain for the production of butyl acetate as a major acetate ester (Nyanga *et al*., 2013). Three different *S. fibuligera* strains were evaluated for their effect on the formation of volatile and non-volatile metabolites in rice wine; thus, facilitating their use in brewing makgeolli, a traditional Korean rice wine (Son *et al*., 2018). Some volatile metabolites derived from phenylalanine, such as phenethyl alcohol, phenethyl acetate, and ethylphenyl acetate, were predominantly found in the culture supernatant of *S. fibuligera* KJJ81, a dominant isolate from wheat-based Nuruk (Lee *et al*., 2018). In a recent study, an *S. fibuligera* strain with elevated ester production capacities was used as a mixed fermentation starter combined with *S. cerevisiae* to improve the flavor profile of Xiaoqu liquor, a type of distilled spirit consumed in China, by increasing the ethanol and ester content (Su *et al*., 2020).

In the present study, we identified 12 multiple *ATF* orthologues (*SfATF*), encoding putative alcohol-*O*-acetyltransferases, in the heterozygous diploid genome of *S. fibuligera* KJJ81, and validated their enzymatic activity and subcellular localization at the LDs by expressing the *SfATF* genes in the *S. cerevisiae* heterologous host.

Materials and Methods

Strains, plasmids, and cultivation conditions

The *S. cerevisiae* strain used for *SfATF* expression was BY4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*), and the plasmids

Table 1. Plasmids used in the present study

Plasmid	Description	Source
YEGa-MCS	2 μ origin-based expression vector with the *GAL10* promoter, *GAL7* terminator, a *URA3* marker, and a multicloning site (MCS)	Choi *et al*. (2013)
YEpUG-ScATF1-6His	YEGa for expression of ScATF1-6His	This study
YEpUG-SfATF(A)1N-6His	YEGa for expression of SfATF(A)1N-6His	This study
YEpUG-SfATF(A)2-6His	YEGa for expression of SfATF(A)2-6His	This study
YEpUG-SfATF(A)3-6His	YEGa for expression of SfATF(A)3-6His	This study
YEpUG-SfATF(A)4-6His	YEGa for expression of SfATF(A)4-6His	This study
YEpUG-SfATF(A)5-6His	YEGa for expression of SfATF(A)5-6His	This study
YEpUG-SfATF(A)6-6His	YEGa for expression of SfATF(A)6-6His	This study
YEpUG-SfATF(B)1-6His	YEGa for expression of SfATF(B)1-6His	This study
YEpUG-SfATF(B)2-6His	YEGa for expression of SfATF(B)2-6His	This study
YEpUG-SfATF(B)3-6His	YEGa for expression of SfATF(B)3-6His	This study
YEpUG-SfATF(B)4-6His	YEGa for expression of SfATF(B)4-6His	This study
YEpUG-SfATF(B)5-6His	YEGa for expression of SfATF(B)5-6His	This study
YEpUG-SfATF(B)6-6His	YEGa for expression of SfATF(B)6-6His	This study
YCpHT	Centromere origin-based expression vector with the *TEF1* promoter and a *HIS3* marker	This study
pDLMOX-yEGFP	Expression vector for yeast enhanced GFP under control of the MOX promoter	Sohn *et al*. (2012)
YCpHT-EGFP	YCpHT for expression yEGFP	This study
YCpHT-ScATF1-EGFP	YCpHT for expression ScATF1-yEGFP	This study
YCpHT-SfATF(A)2-EGFP	YCpHT for expression SfATF(A)2-yEGFP	This study
YCpHT-SfATF(B)2-EGFP	YCpHT for expression SfATF(B)2-yEGFP	This study
YCpHT-SfATF(B)6-EGFP	YCpHT for expression SfATF(B)6-yEGFP	This study
pERGmDsRed	2 μ origin-based expression vector with the *PGK1* promoter and a *LEU2* marker for ScERG6-DsRed	Lin and Wheeldon (2014)

Table 2. ***Saccharomycopsis fibuligera*** **ORFs annotated as AATases with homology to ScAtf1 protein**

ORF ID	Annotation (Pfam domain)	Identity to ScAtf1p (%)	Gene name	Expected gene product size (kDa)
KJJ81A 4G012700	Alcohol acetyltransferase (PF07247)	21.2	*SfATF(A)1N*	32.23
KJJ81A4G012800	Alcohol acetyltransferase (PF07247)	27	*SfATF(A)1C*	16.28
KJJ81A2G089500	Alcohol acetyltransferase (PF07247)	18.1	*SfATF(A)2*	60.94
KJJ81A5G003500	Alcohol acetyltransferase (PF07247)	17.1	*SfATF(A)3*	77.99
KJJ81A2G089400	Alcohol acetyltransferase (PF07247)	16.8	*SfATF(A)4*	46.42
KJJ81A3G017700	Alcohol acetyltransferase (PF07247)	15.4	*SfATF(A)5*	54.78
KJJ81A2G066500	Alcohol acetyltransferase (PF07247)	14.7	*SfATF(A)6*	Without intron: 61.49 With intron: 6.38
KJJ81B4G012200	Alcohol acetyltransferase (PF07247)	17.1	*SfATF(B)1*	57.09
KJJ81B2G087000	Alcohol acetyltransferase (PF07247)	17.1	*SfATF(B)2*	60.94
KJJ81B5G003500	Alcohol acetyltransferase (PF07247)	14.9	*SfATF(B)3*	76.78
KJJ81B2G086900	Alcohol acetyltransferase (PF07247)	15.5	*SfATF(B)4*	57.42
KJJ81B3G017600	Alcohol acetyltransferase (PF07247)	18.4	*SfATF(B)5*	55.22
KJJ81B2G064500	Alcohol acetyltransferase (PF07247)	13.3	*SfATF(B)6*	Without intron: 61.27 With intron: 4.51

used in this study are listed in Table 1. Yeast cells were grown in YPD (1% yeast extract, 2% bacto peptone, 2% glucose) or synthetic complete medium (SC; 0.67% yeast nitrogen base without amino acids, 2% glucose, 1× drop-out amino acid mixture) at 30°C. Yeast transformation was carried out using the LiAc/SS Carrier DNA/PEG method (Gietz and Schiestl, 2007). For selection of Leu^+, Ura^+, or His^+ transformants, synthetic complete medium without leucine (SC-LEU), uracil (SC-URA), or histidine (SC-HIS) was used. To analyze the expression of His-tagged intracellular SfAtf proteins, the recombinant *S. cerevisiae* cells were cultivated in selective SCG-URA medium (0.67% yeast nitrogen base without amino acids, 2% galactose, 1% glucose, and 0.77 g/L drop-out amino acid mixture supplemented with all required amino acids without uracil).

Construction of *S. cerevisiae* expression vectors for *S. fibuligera* Atf proteins

Each open reading frame (ORF) of the *SfATF* genes (Table 2) was amplified using PCR and the primer sets listed in Supplementary data Table S1 from the genomic template of *S. fibuligera* KJJ81 (Choo *et al.*, 2016). The PCR products containing *SfATF* ORF with a C-terminal 6His tag were digested with XhoI/NotI and cloned into YEGa-MCS, generating a set of YEpUG-SfATF expression vectors (Table 1). The ORF of *ScATF1* was amplified using PCR and the genomic template of *S. cerevisiae* BY4742 as a His-tagged form using the primer sets listed in Supplementary data Table S1. Subsequently, it was subcloned into the NotI/SalI site of YEGa-MCS. The resultant YEpUG-ATF-6His plasmids express His-tagged Atf proteins under the control of the *GAL10* promoter and *GAL7* terminator. The *S. cerevisiae* expression vectors expressing *S. fibuligera* Atf-GFP fusion proteins was constructed as follows: The ORF of yEGFP was amplified from pDLMOX-yEGFP using PCR and appropriate primers (Supplementary data Table S1). The DNA fragments of *ScATF1*, *SfATF(B)2*, and *SfATF(B)6* were amplified using PCR from YEpUG-ScATF1-6His, YEpUG-SfATF(B)2-6His, and YEpUG-SfATF(B)6-6His templates, respectively, and the primer sets corresponding to each gene (Supplementary data Table S1). The ATF-yEGFP fusion constructs were designed to carry five glycine linkers between ATF and yEGFP, and the fusion PCR products were cloned into the SpeI/SalI site of the YCpHT plasmid, which is a CEN-based expression vector with a *TEF1* promoter, a *GAL7* terminator, and an *HIS3* marker, generating YCpHT-ScATF1-EGFP, YCpHT-SfATF(B)2-EGFP, and YCpHT-SfATF(B)6-EGFP (Table 1). The resulting plasmids were used to transform the *S. cerevisiae* BY4742 strain. The pERGmDsRed plasmid containing the ScErg6-DsRed and *ScLEU2* marker (kindly provided by Prof. Ian Wheeldon, University of California Riverside) was used to express an LD marker ScErg6 protein.

Western blot analysis of SfAtf protein expression

For preparation of the cytosolic fraction, the yeast cells were lysed by vortexing cell pellets in TNE lysis buffer (50 mM Tris-HCl; pH 7.5, 150 mM NaCl, 5 mM EDTA, protease inhibitor cocktail [Sigma Aldrich], 1 mM PMSF). The protein samples were separated using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot was performed using the anti-His antibody (Santa Cruz Biotechnology) and the β-actin (Abcam) antibody at a dilution of 1:200 and 1:1,000, respectively.

HS-SPME GC/MS (Headspace-solid phase microextraction coupled to gas chromatography/mass spectrometry) analysis

Yeast cells were inoculated to be adjusted to an optical density at 600 nm of 0.5 (OD_{600} = 0.5) in SCG-URA medium and incubated for 24 h. The obtained yeast culture supernatants were transferred into headspace vials (Sigma Aldrich) and analyzed using the splitless mode of the HS-SPME GC/MS (7820A/5977E MSD, Agilent Technologies) equipped with a DB-wax column (50 m × 200 μm × 0.2 μm; Agilent Technologies) operating at a flow rate of 1 ml/min of helium. The sample equilibrium process was performed for 5 min at 50°C. The volatile components were absorbed onto a 50/30 μm divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fiber for 30 min and desorbed into the injection port for 2 min at 250°C. The MS was analyzed in the SCAN mode (*m*/*z* 33-200) with an ionization energy of 70 eV. Oven temperature was programed for 5 min at 40°C increasing to 150°C

at a speed of 5°C/min and to 200°C at a speed of 7°C/min. The 200°C temperature was held of 10 min. Volatile compounds were identified using a library program (the National Institute of Standards and Technology, NIST Mass Spectral Search Program Version 2.0 g). Identified volatile compounds were quantified in SCAN mode using total ion chromato-

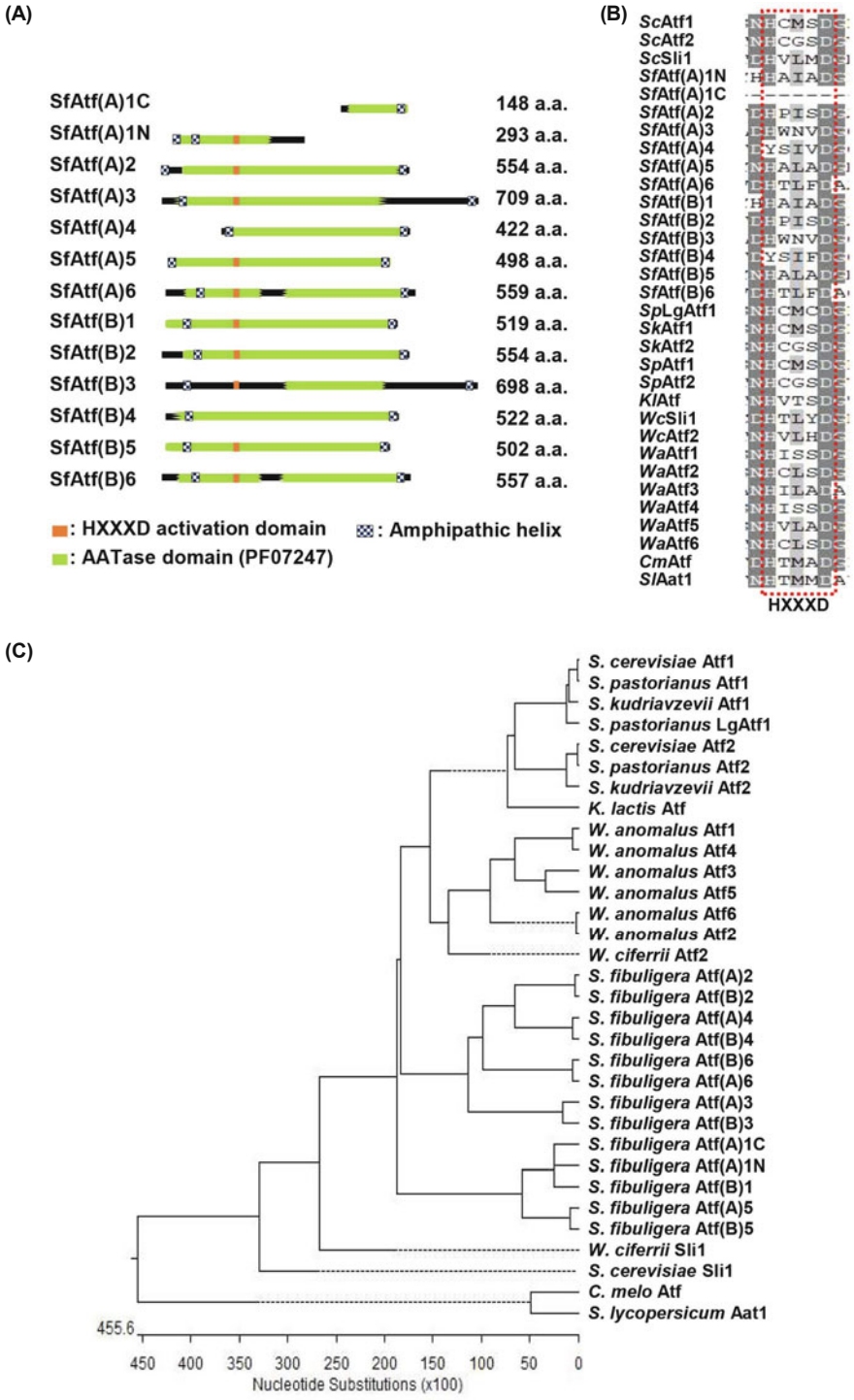

Fig. 1. The domain structure and phylogenetic tree analysis of SfAtf proteins. (A) Domain structure of putative SfAtf proteins. The position of AATase domain (PF07247) was delineated by analyzing the protein sequences of the putative SfAtf orthologs for Pfam matches at the Pfam database (http://pfam.xfam.org). (B) Multiple sequence alignment of the conserved activation domain (HXXXD) among yeast Atf proteins. The multiple sequence alignment was constructed using the CLUSTALW method in the DNASTAR MegAlign software and shaded using GeneDoc (https://genedoc.software.informer.com/). (C) Phylogenetic tree based on amino acid sequences of AATase orthologs from various yeast and plant species. The phylogenetic tree was constructed using the neighbor joining (NJ) algorithm built using the MEGA7 software. GenBank accession numbers of the AATase orthologs are listed in Supplementary data Table S3.

gram (TIC) on an arbitrary scale. The analysis of volatile compounds using HS-SPME GC/MS was carried out in biological duplicate.

Helix wheel analysis of N-/C-terminus of SfAtf proteins

Protein structures of SfAtf proteins were predicted using the intense mode of Phyre 2, a protein structure prediction tool (http://www.sbg.bio.ic.ac.uk/phyre2), which uses the alignment of hidden Markov models via HHsearch (Söding, 2005) and a new *ab initio* folding simulation called Poing (Jefferys *et al.*, 2010). The N- and C-terminal 18 sequences of SfAtf proteins were analyzed for helix wheel properties and secondary structures using HELIQUEST (http://heliquest.ipmc.cnrs.fr/) and PEP-FOLD 3.5 (https://mobyle.rpbs.univ-paris-diderot.fr/), respectively.

Microscopic analysis of Atf-GFP localization

Yeast cells harboring the ScERG6-DsRed- and ATF-yEGFP-expressing plasmids were cultivated in SC-LEU-HIS medium until the late log phase and were subsequently cultured for an additional 48 h in oleic acid medium containing 0.67% yeast nitrogen base, 0.1% yeast extract, 0.2% Tween 80, and 0.1% oleic acid to promote LD synthesis (Lin and Wheeldon, 2014). Following harvest, cells were fixed with 4% paraformaldehyde (pH 7) for 20 min at 4°C with rotation in the dark. The fixed cells were adjusted to an OD_{600} of 1.0 in PBS and observed using an Eclipse Ti-E fluorescence microscope (Nikon) equipped with Nikon DS-Qi2 camera and Plan Apo VC 100× Oil DIC N2 (NA 1.4) lens. Images were processed using the NIS-elements microscope imaging software (Nikon).

Results

Identification and structural domain analysis of *S. fibuligera ATF* gene family

Our previous study on the *de novo* whole genome sequencing of *S. fibuligera* KJJ81 (GenBank accession nos. CP012809–CP012822) revealed that the ~38 Mb genome of this yeast strain is a heterozygous diploid, consisting of subgenomes A and B that share 88.1% of nucleotide identity. A high-quality annotation of *S. fibuligera* KJJ81 genome, employing *ab initio* and evidence-driven gene prediction methodology, predicted a non-redundant set of 12,135 protein-coding genes, and orthologous gene families were assigned against the Pfam database using HMMPfam (Choo *et al.*, 2016). Similarity search for a *S. cerevisiae ATF1* gene orthologues throughout the entire annotated genome of *S. fibuligera* KJJ81 was initially performed based on the presence of Pfam domain PF07247 that is found in a number of AATases, leading to the identification of 13 ORFs (Table 2). The putative *S. fibuligera* ATF proteins display overall low sequence identities with *S. cerevisiae* Atf1p (between 13.3 and 27.0%), and the number in the name of SfAtf proteins in subgenome A was assigned according to the extent of identity with *S. cerevisiae* Atf1p. Subsequently, the name of SfAtf proteins in subgenome B was assigned according to that of SfAtf proteins in the same chromosome. Thus, both *S. fibuligera* subgenome A and B contained six *ATF* orthologues: *SfATF1* localized on chromosome 4, *SfATF2* on chromosome 3, *SfATF5* on chromosome 5, *SfATF4* on chromosome 2, *SfATF5* on chromosome 3, and *SfATF6* on chromosome 2. The *SfATF* genes from subgenome A and B, a pair of *SfATF(A)* and *SfATF(B)*, encode proteins sharing amino acid sequence identity of 80.5–93.9% each other (Supplementary data Table S2), reflecting the heterozygous diploid genome of *S. fibuligera* KJJ81 generated by hybrid formation between two different *S. fibuligera* subspecies (Choo *et al.*, 2016). Notably, the sequence identities among the six SfAtf proteins are relatively low, ranging between 12.0 and 45.8%. Intriguingly, the ORF of *SfATF1* in subgenome A was split into two short ORFs, *SfATF(A)1N* and *SfATF(A)1C*, due to the presence of a stop codon in the middle of the putative ORF of *SfATF(A)1* (Fig. 1A). All *S. fibuligera* Atf proteins, except SfAtf(A)4p and SfAtf(B)4p, contained the activation domain (HXXXD), which is commonly found in Atf orthologues from various yeast (Van Laere *et al.*, 2008; ter Veld *et al.*, 2013) and fruit species (Galaz *et al.*, 2013; Goulet *et al.*, 2015) (Fig. 1B).

Phylogenetic analysis of Atf proteins was carried out across *S. fibuligera*, *S. cerevisiae*, other *Saccharomyces sensu stricto* species including *S. pastorianus* and *S. kudriavzevii*, non-*Saccharomyces* yeast species including *K. lactis*, *W. anomalus*,

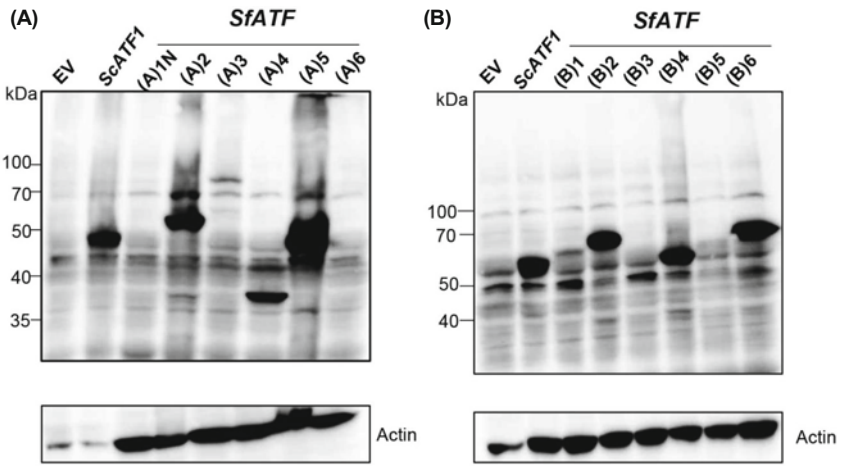

Fig. 2. Overexpression of SfAtf proteins in the heterologous host *S. cerevisiae*. Western blotting of the cell lysates of *S. cerevisiae* expressing His-tagged SfAtf(A) (left) and SfAtf(B) (right) proteins was performed using anti-His antibody. The *S. cerevisiae* cells expressing the empty vector (EV), *ScATF1*, and *SfATF* genes were cultured in SCG-URA medium containing 1% glucose and 2% galactose to induce the expression of *SfATF* under the *GAL10* promoter for 24 h.

and *W. ciferrii*, as well as two plant species including *Cucumis melo* and *Solanum lycopersicum*, which showed very low amino acid sequence identity to other known Atf genes of other species but have functional enzymatic activity (Fig. 1B and Supplementary data Table S2). The phylogenetic tree indicated that SfAtf2, 3, 4, and 6 proteins are grouped together but distinctive from other Atfp orthologues, which diverged earlier from the common ancestor (Fig. 1C). Although SfAtf1p and StAtf5p appeared to be more closely associated with other yeast Atf orthologs, they are separately grouped from *Saccharomyces* species and non-*Saccharomyces* species in the phylogenetic tree, indicating that Atf proteins are generally grouped within species. We included the *S. cerevisiae N*-acetyltransferase Sli1p (encoded by SLI1/YGR212w) (Momoi *et al*., 2004) and the *W. ciferrii N*-acetyltransferase Sli1p (encoded by NCBI gene ID 23469349) (ter Veld *et al*., 2013), which exhibits weak similarities to *S. cerevisiae* Atf1p and Atf2p (14.8 and 15.2% identity, 17.5 and 16.8% identity, respectively), in the phylogenetic analysis. Separation of yeast Sli1 proteins as an outgroup of the clade including all the yeast Atf proteins indicates that despite their very low sequence identities to *S. cerevisiae* Atf1p, the putative *S. fibuligera* Atf proteins belong to the alcohol-*O*-acetyltransferases family involved in volatile ester synthesis.

Volatile aroma profiling of the recombinant *S. cerevisiae* expressing SfAtf proteins

To verify the function of SfAtf proteins {except SfAtf(A)1Cp} as AATases, all *SfATF* genes obtained from the *S. fibuligera* genomic DNAs were expressed under the *GAL10* promoter on the 2 μ-based vector in the heterologous host *S. cerevisiae* BY4742. The expression of the SfAtf proteins, tagged with the C-terminal His-epitope, was analyzed using western blotting with an anti-His antibody (Fig. 2). While the signals corresponding to SfAtf(A)1Np, SfAtf(A)6p, SfAtf(B)3p, and SfAtf(B)5p were not noticed, the protein bands with the predicted size of SfAtf(A)2p, SfAtf(A)3p, SfAtf(A)4p, SfAtf(A)5p, SfAtf(B)1p, SfAtf(B)2p, SfAtf(B)4p, and SfAtf(B)6p were detected in the cell lysate of recombinant *S. cerevisiae* strains (Fig. 2 and Table 2). It was reported that *S. fibuligera* genome contain introns with average length of 205 bp at very low frequency; the number of average introns per gene is 0.23 (Choo *et al*., 2016). Among twelve *SfATF* genes, only the *SfATF(A)6* and *SfATF(B)6* genes contains an intron with a stop codon. Without splicing of the intron, a very short peptide of 4.51 kDa would be generated as a translational product of the *SfATF(B)6* gene, whereas after correct splicing, a protein of 61.27 kDa would be expected as a translational product (Supplementary data Fig. S1). The protein size observed following western blotting was congruent with the size expected following intron splicing (Fig. 2). The result indicated that the *S. fibuligera* intron with the conserved consensus sequences of splicing observed in the *SfATF(B)6* genomic DNA fragment was correctly spliced to generate intron-less mature mRNA in the heterologous host *S. cerevisiae*. SfAtf(A)3p and SfAtf(B)1p were expressed at a low level, while SfAtf(A)4p was expressed as a truncated protein with a smaller than expected size. Intriguingly, the SfAtf proteins highly expressed in *S. cerevisiae*, including SfAtf(A)2p, SfAtf(A)5p, SfAtf(B)2p, SfAtf(B)4p, and SfAtf(B)6p, were found to show higher endogenous transcript levels in the native host *S. fibuligera* under normal culture condition than those of the non-expressed proteins (Supplementary data Fig. S2).

The activity of alcohol-*O*-acetyltransferase was examined by HS-SPME GC/MS analysis of the culture supernatant of the *S. cerevisiae* recombinant cell, which were cultivated in SCG-URA containing 2% galactose and 1% glucose for 24 h (Fig. 3; Supplementary data Fig. S3 and Table S4). After consuming glucose as a preferential C-source for their growth,

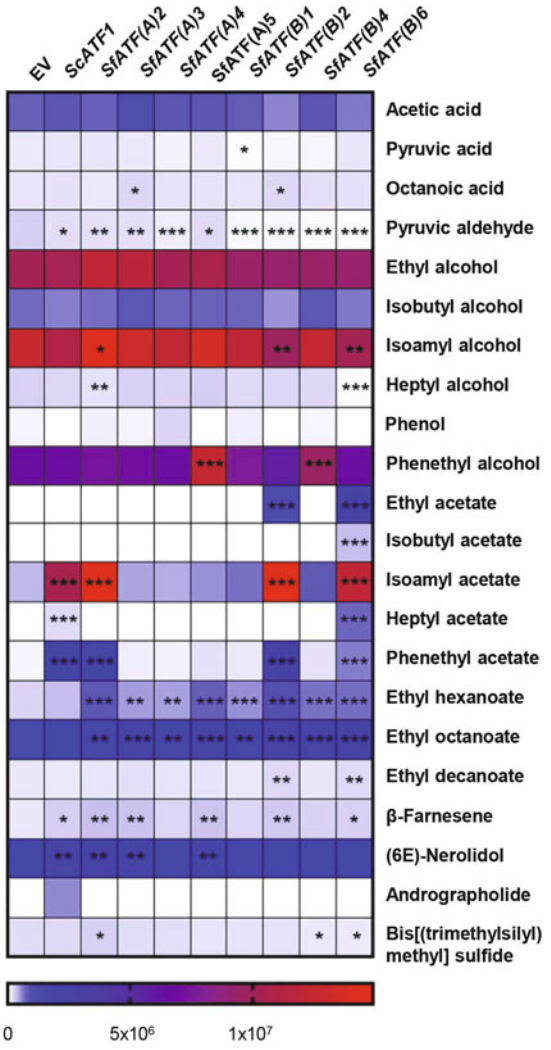

Fig. 3. Heatmap analysis of volatile compounds generated by the recombinant *S. cerevisiae* expressing SfAtf proteins. The yeast volatile compounds cultured in SCG-URA medium for 24 h were analyzed using HS-SPME GC/MS. Heatmap shows the relative abundance of volatile compounds in the culture supernatants generated by the recombinant *S. cerevisiae* strains, expressing the empty vector (EV), the *ScATF1* gene, and the *SfATF* genes, compared to that of the control medium (SCG-URA) without yeast cultures. Each sample was compared to that of the *S. cerevisiae* strain expressing an empty vector (EV) within compounds using a one-way ANOVA and the Dunnett's multiple comparisons test. Statistical analyses were performed using Graphpad Prism version 7.00 (GraphPad Software). The observed differences were considered statistically significant for P-values < 0.05. * indicates the significant differences between control medium and each yeast inoculated medium (P value < 0.033 [*], 0.002 [**], 0.001 [***]).

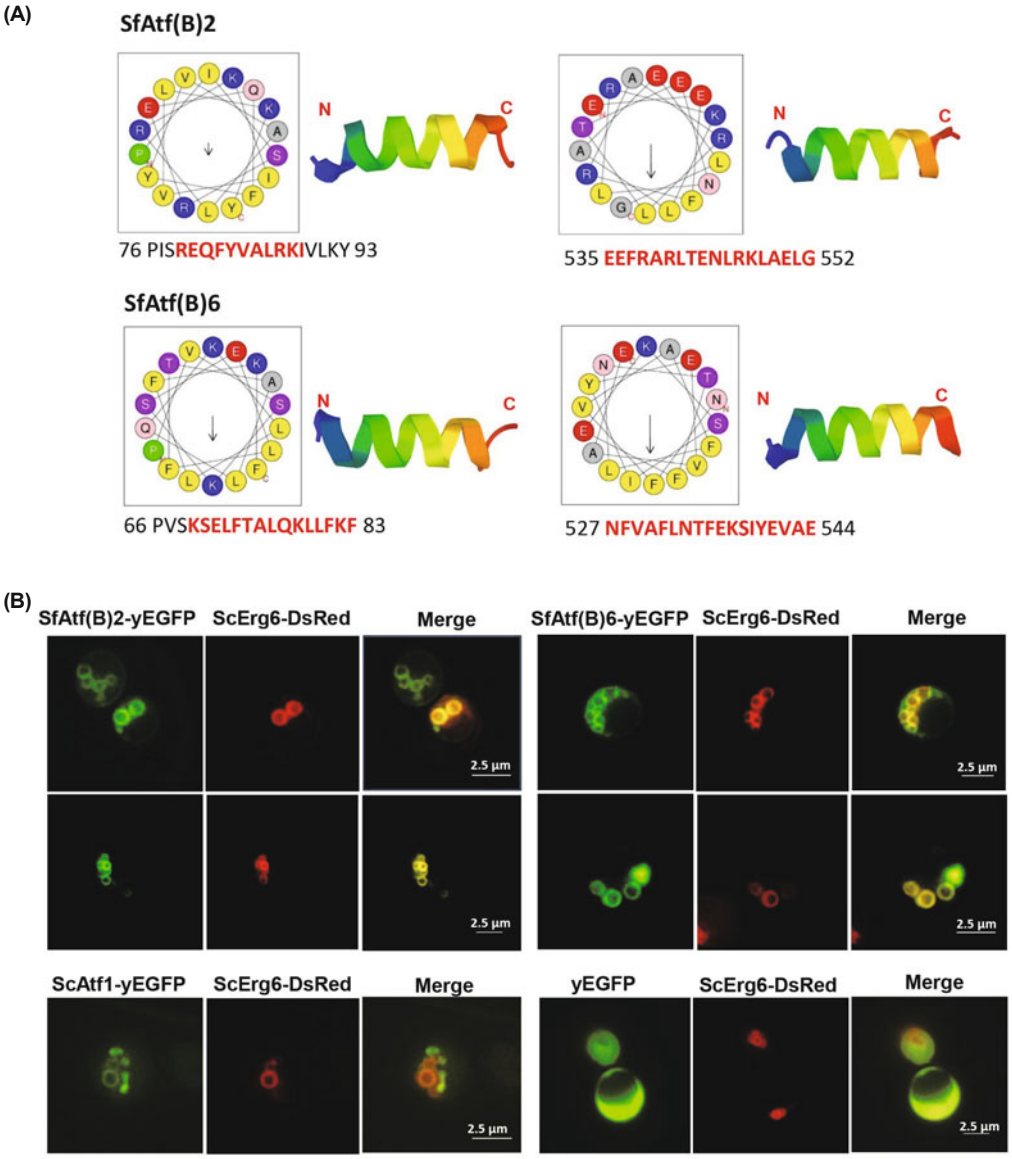

Fig. 4. Subcellular localization of SfAtf proteins. (A) Helix wheel analysis and predicted peptide structure models of N- and C-termini of SfAtf(B)2 (upper panel) and SfAtf(B)6 (lower panel). Hydrophobic residues are represented in yellow, neutral in gray, basic in blue, and acidic in red. The predicted peptide structure is shown as a rainbow, with the N-terminus in blue and the C-terminus in red. The helix wheel and the secondary structure of SfAtf proteins were drawn using HELIQUEST and PEP-FOLD3, respectively. (B) Fluorescent microscopic analysis of SfAtf(B)2-yEGFP and SfAtf(B)6-yEGFP expressed in *S. cerevisiae*. Fluorescent microscopic images were obtained from the yeast cells co-expressing Atf-yEGFP (FITC, Excitation – 480 ± 30, Emission – 535 ± 45) and the LD marker ScErg6-DsRed (TRITC, Excitation – 540 ± 25, Emission – 605 ± 55). The signals of yEGFP and DsRed are shown in green and red, respectively. Scale bar = 2.5 μm.

yeast cells used galactose as a subsequent C-source and as an inducer of the *GAL10* promoter to express recombinant Atf proteins in the SCG-URA medium. Our preliminary flavor profile analysis at 12 h, 24 h, and 48 h showed the highest levels of volatile compounds at 24 h cultivation (data not shown). The heatmap showing the relative amounts of volatile compounds compared to control medium SCG-URA indicated the formation of acetate esters from high alcohols, such as isoamyl alcohol and phenethyl alcohol, through the action of *S. cerevisiae* and *S. fibuligera* Atf proteins. The production of isoamyl and phenethyl acetate was detected in the *S. cerevisiae* strains expressing SfAtf(A)2p, SfAtf(B)2p, and SfAtf(B)6p at levels that were comparable with the *S. cerevisiae* strains expressing ScAtf1p. Notably, the *S. cerevisiae* strain expressing SfAtf(B)6p generated a smaller amount of phenethyl acetate compared to other strains, while producing higher levels of heptyl and isobutyl acetate than the other recombinant *S. cerevisiae* strains. Furthermore, the increased level of ethyl acetate was detected only in the *S. cerevisiae* strains expressing SfAtf(B)2p and SfAtf(B)6p, indicating that the SfAtf proteins show alcohol *O*-acetyltransferase activity with distinctive substrate specificities. Although the SfAtf(B)4 was highly expressed (Fig. 2), it did not show AATase activity (Fig. 3), which is consistent with the absence of the HXXXD

consensus sequence essential for acetyltransferase activity (Fig. 1B). The SfAtf(A)5p showed an interesting aroma profile in which the level of phenethyl alcohol is increased while no phenethyl acetate was generated (Fig. 3). This might indicate that SfAtf(A)5p has lost the acetate esters synthesis activity despite the presence of the HXXXD consensus sequence. Production of any acetate esters was not detected for the *S. cerevisiae* strains expressing SfAtf(A)3p and SfAtf(B)1p, which might be explained by their extremely low level of expression (Figs. 2 and 3). Overall, our data revealed that while the volatile aroma profile generated by SfAtf(A)2p, SfAtf(B)2p, and SfAtf(B)6p were generally similar to those generated by ScAtf1, the SfAtf proteins exhibited a distinctive AATase activity with different preference for their substrates.

Subcellular localization of SfAtf proteins at lipid droplets

Previous studies showed that *S. cerevisiae* Atf1p is localized to the ER and LDs via the amphipathic domains at the N- and C-termini (Verstrepen *et al*., 2004; Lin and Wheeldon, 2014). Among SfAtf(A)2p, SfAtf(B)2p, and SfAtf(B)6p that were confirmed to be expressed in *S. cerevisiae* and exhibit AATase activity, we analyzed the whole protein structures of SfAtf(B)2p and SfAtf(B)6p, using the intense mode of Phyre2, which is a suite of tools to predict and analyze structure of proteins even without known templates via *ab initio* modeling (Kelley *et al*., 2015). In our initial localization analysis, SfAtf(B)2p and SfAtf(B)6p were chosen because SfAtf(A)2p shares 93.9% amino acid sequence identity with SfAtf(B)2p (Supplementary data Table S2). The N- and C-terminal sequences of SfAtf proteins, which were predicted by Phyre2 to be exposed at the surface of the whole protein structure and likely to contain α-helices, were further subjected to in-depth analyses. The physicochemical properties and amino acid composition of an alpha helical structure in the N- and C-terminal sequences was analyzed by using an alpha-helix analysis tool HELIQUEST (Gautier *et al*., 2008) and the secondary structure of the terminal sequences was confirmed by using a *de novo* peptide structure prediction tool PEP-FOLD3 (Lamiable *et al*., 2016). The 76–93 and 535–552 residues of SfAtf(B)2p as well as 66–83 and 527–544 residues of SfAtf(B)6p were predicted to be amphipathic helix; thus, indicating a potential association with LD (Fig. 4A). To validate the localization of SfAtf(B)2 and SfAtf(B)6 proteins at the LDs, we further analyzed the subcellular localization of the SfAtf(B)2-GFP and SfAtf(B)6-GFP fusion proteins in *S. cerevisiae*. The Atf proteins fused with the yeast enhanced GFP (yEGFP) at their C-terminus, and ScAtf1-yEGFP, SfAtf(B)2-yEGFP, and SfAtf(B)6-yEGFP were constructed and expressed under the control of the *ScTEF1* promoter in a CEN plasmid. Following culturing in oleic acid medium for 48 h, both SfAtf(B)2-yEGFP and SfAtf(B)6-yEGFP proteins were observed to co-localize with ScErg6-DsRed, a co-expressed LD marker (Lin and Wheeldon, 2014). The green signals of SfAtf(B)2-yEGFP and SfAtf(B)6-yEGFP proteins were clearly detected at LDs and merged with signals of ScErg6-DsRed. A minor fraction of Atf-yEGFP fusion constructs did not co-localize with LDs. It is speculated that some portion of Atf-yEGFP fusion constructs were subjected to proteolytic cleavage at the linker region, generating only yEGFP, or to protein degradation, generating truncated Atf-yEGFP fusion constructs lacking N-/C-terminal part. Such degradation products of Atf-yEGFP fusion constructs cannot be co-localized with LDs due to the loss of amphipathic helix terminus. We detected a substantial protein degradation in the western blot analysis of SfAtf-yEGFP fusion constructs expressed in *S. cerevisiae* (Supplementary data Fig. S4). Furthermore, similar results were obtained for ScAtf1-yEGFP, while the yEGFP control showed dispersed fluorescence in the cytosols (Fig. 4B). Altogether, the results suggest that SfAtf(B)2 and SfAtf(B)6 proteins localize to lipid particles, which is consistent with the presence of their amphipathic helices at the N- and C-termini. After validating the predicted LD-associated localization of SfAtf(B)2p and SfAtf(B)6p by GFP fusion analysis, we extended the *in silico* structural analysis to all the other SfAtf proteins. Further analysis of the N- and C-termini of the other SfAtf(A) and SfAtf(B) proteins predicted that most *S. fibuligera* Atf orthologs exhibit amphipathic helices at both terminals to different extents (Supplementary data Fig. S5), indicating that all *S. fibuligera* Atf proteins might be associated with LDs.

Discussion

The traditional yeast *S. cerevisiae* has been the routinely used yeast for food fermentations. Non-*Saccharomyces* yeasts, which are naturally present in un-inoculated and spontaneous fermentations, might provide a means for increasing the aroma and flavor diversity in fermented beverages. Thus, there is a growing interest in non-conventional yeast species that can generate diversity in the aroma profiles of the end products (Varela, 2016). In general, the Ehrlich pathway is expressed at a higher level in several non-conventional yeasts than in *Saccharomyces* species, and all three fusel alcohols derived from branched amino acids are produced at much higher concentrations; thus, non-conventional yeast species generate increased levels of acetate ester (Aslankoohi *et al*., 2016; Gamero *et al*., 2016; van Rijswijck *et al*., 2017). In the present study, we demonstrate the high potential of the amylolytic yeast *S. fibuligera* as a producer of high-level acetate esters and source of novel *ATF* genes for diverse flavor production.

The *ATF* genes, encoding alcohol acetyltransferases, in various yeast species have attracted interest due to their biotechnological potential for the production of compounds that are aromatic. The *S. cerevisiae stricto* yeasts have two genes coding for AATases, highly similar to the *S. cerevisiae ATF1* and *ATF2*. In contrast, the more distantly related yeasts, such as *S. castelli, C. glabrata*, *K. waltii*, and *K. lactis* have only one orthologue in their genome (Van Laere *et al*., 2008). However, a recent genome sequencing analysis of *W. anomalus* revealed the presence of six AATases (Schneider *et al*., 2012). We bioinformatically identified six *ATF* orthologs per haploid genome in *S. fibuligera* KJJ81 (Fig. 1 and Table 2). Furthermore, all putative SfAtf proteins, except SfAtf(A)4p and SfAtf(B)4p, contain the conserved HXXXD (G) active site motif that is common to CoA-dependent O-acyltransferases and the plant AATs within the BAHD superfamily in particular (D'Auria, 2006). The presence of multiple *ATF* genes might partly contribute to the increased levels of acetate ester

production in non-conventional yeast species, including *S. fibuligera*. While the *S. cerevisiae ATF1* gene is only expressed in the absence of oxygen (Yoshimoto *et al*., 1998), our previous RNA-Seq data (Choo *et al*., 2016) revealed that the *SfATF* genes, except *SfATF(A)6*, are expressed at low or moderate levels under aerobic cultivation conditions in *S. fibuligera*, which is a Crabtree-negative yeast (Supplementary data Fig. S2). The expression levels of *SfATF* were not noticeably changed at lower glucose levels (0.1% glucose) and lower temperature (25°C), compared to the normal growth condition (YPD containing 2% glucose, at 37°C), indicating the lack of glucose repression and temperature effect at the transcriptional level. However, the expression of several *SfATF* genes was highly enhanced under sulfur-limited conditions, suggesting that the *SfATF* gene expression might be subject to catabolite transcriptional repression in the presence of sulfur. The RNA-Seq data also indicated that the *SfATF(B)* transcription levels are overall higher than those of *SfATF(A)* genes, as previously reported on the differential gene expression profiles between the two subgenomes of *S. fibuligera* KJJ81 (Choo *et al*., 2016).

A previous study on heterologous expression of AATases in two common microbial hosts, *Escherichia coli* and *S. cerevisiae*, reported that all AATases tested were expressed as intracellular aggregates with low enzymatic activity in *E. coli*, indicating that *S. cerevisiae* is more suitable host for analysis of intracellular localization, enzymatic activity, and expression level (Zhu *et al*., 2015). We overexpressed all *SfATF* genes in the heterologous host *S. cerevisiae* and showed that the recombinant *S. cerevisiae* strains overexpressing *SfATF(A)2*, *SfATF(B)2*, and *SfATF(B)6* increased ester synthesis at a comparable level with the *S. cerevisiae ATF1* overexpression (Figs. 2 and 3). This strongly supports that *SfATF(A)2*, *SfATF(B)2*, and *SfATF(B)6* encode functional AATases that play a major role in acetate ester synthesis. However, we do not yet know why *SfATF(A)1N, SfATF(A)3, SfATF(A)6, SfATF(B)1, SfATF(B)3*, and *SfATF(B)5* were expressed at a low level or not expressed at all in *S. cerevisiae*. Previous studies also reported encountering unknown difficulties in overexpressing recombinant *S. cerevisiae* Atf2p in *E. coli* (Zhu *et al*., 2015) and in *S. cerevisiae* (Nancolas *et al*., 2017). Notably, the SfAtf proteins were predicted to carry the N- and C-amphipathic helices and shown to be associated with LDs (Fig. 4), as observed for *S. cerevisiae* Atf1 and Atf2 proteins. On contrary, the AATases from *K. lactis* and *W. anomalus*, which do not have the terminal amphipathic helices, were localized to the cytoplasm when expressed in *S. cerevisiae* (Lin and Wheeldon, 2014). Thus, SfAtf proteins are the first example of non-*Saccharomyces* AATases to be localized at the LDs.

Saccharomyces cerevisiae Atf1 is known to be promiscuous about alcohol co-substrates although the acyltransferase activity is specific for acetyl-CoA. Additionally, *in vitro*, Atf1 exhibits an efficient thioesterase targeting medium-chain-length acyl-CoAs (Nancolas *et al*., 2017). Compared to the reference strain with endogenous *ATF* genes, the more enhanced phenethyl and isoamyl acetate production in *S. cerevisiae* strains that harbored *ATF1* and *ATF2* genes from *S. kudriavzevii* and *S. uvarum* was reported; thus, indicating the distinct properties of Atf enzymes among different yeast species (Stribny *et al*., 2016). Our data of volatile aroma profiling suggested that the substrate specificity of SfAtf(A)2 is similar to that of ScAtf1; however, SfAtf(B)2 and SfAtf(B)6 showed a broader substrate specificity (Fig. 3). Such distinct enzymatic properties, along with the presence of multiple *ATF* genes and different gene expression patterns, could give rise the differences in acetate ester formation during fermentation among different yeast species. Further biochemical analysis of purified SfAtf proteins would be required to elucidate their enzymatic properties with defined substrate specificity.

Small to medium volatile esters are valuable natural food additives as aroma and flavor compounds in fermented beverages and foods. Thus, the enzymes responsible for ester synthesis are targets for the metabolic engineering of cellular 'factories' intended to produce fragrances, industrial solvents, fine chemicals, pharmaceuticals, and renewable biofuels (Zhu *et al*., 2015; Kruis *et al*., 2017). Particularly, *S. cerevisiae* Atf1 has been exploited as a component for synthetic biology applications to produce the industrial solvent and food additive volatile esters in *E. coli* (Rodriguez *et al.,* 2014; Tashiro *et al.,* 2015a). With the increase of whole genome sequencing of non-conventional yeasts, more *ATF* genes will be newly identified and characterized. The knowledge on the key enzymes for acetate esters is expected to help the scientists better understand the AATase protein family in diverse yeast species. Moreover, the newly isolated *ATF* genes with distinct substrate specificity can be used to generate more diverse volatile esters at higher levels.

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Conflict of Interest

The authors declare that there are no conflicts of interest.

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