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Genome-Wide Identification and Biochemical Characterization of Alcohol Acyltransferases for Aroma Generation in *Wickerhamomyces subpelliculosus* Isolates from Fermented Food

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ABSTRACT: The importance of nonconventional yeasts has increasingly been highlighted, particularly for aroma formation in fermented foods. Here, we performed *de novo* whole-genome sequencing of *Wickerhamomyces subpelliculosus*, which produces a variety of volatile flavor compounds, leading to the identification of the alcohol acyltransferase (AATase) family of genes. The genome of *W. subpelliculosus* contains seven AATase genes, encoding alcohol-*O*-acetyltransferases (ATFs) and ethanol acetyltransferase 1 (EAT1) for acetate ester formation, along with ethanol hexanoyl transferase 1 (EHT1) for ethyl ester formation. Among five ATF homologues, only WsATF5 showed acetyltransferase activity toward myriocin, a structural analogue of sphingosine. In contrast, heterologous expression of WsEHT1 and WsEAT1 in *Saccharomyces cerevisiae* promoted the production of ethyl decanoate and ethyl acetate, respectively, supporting their AATase activity. The enzymatic activity analyses revealed the additional alcoholysis activity of WsEAT1 and the thioesterase activity of WsEHT1. Subcellular localization analysis indicated that WsEAT1 was localized in the mitochondria, WsEHT1 in the endoplasmic reticulum and lipid droplets (LDs), and WsATF5 in the LDs. The novel *W. subpelliculosus* AATases could be usefully applied to produce flavor components in various food industries.

KEYWORDS: *Wickerhamomyces subpelliculosus, whole-genome sequencing, volatile ester, alcohol acyltransferase, EAT1, EHT1*

1. INTRODUCTION

Esters are important flavor compounds in many food products. Volatile esters produced by bacteria, yeasts, and fungi during fermentation can be grouped as acetate esters and medium-chain fatty acid (MCFA) ethyl esters.[1,2] Acetate esters, in which the alcohol group from ethanol or higher alcohols is attached to acetate, consist, for example, of ethyl acetate (pear, fruity, and solvent-like aromas), isoamyl acetate (banana aroma), isobutyl acetate (fruity aroma), and phenethyl acetate (2-phenylethyl acetate; rose aroma).[2] MCFA-ethyl esters, in which the alcohol group from ethanol is attached to a MCFA, include ethyl hexanoate (aniseed and apple-like aromas), ethyl octanoate (sour apple aroma), and ethyl decanoate (floral aroma).[2] Ester biosynthesis occurs via four main enzymatic pathways, mediated by the actions of esterases, hemiacetal dehydrogenation (HADH), Baeyer−Villiger monooxygenases (BVMOs), and alcohol acyltransferases (AATases).[3] Reactions catalyzed by esterases and AATases are redox reactions, whereas HADH and BVMOs require NAD(P) and NAD(P)H, respectively.[3] AATases are a large and diverse group of enzymes that can form volatile esters by transferring acyl chains from acyl-coenzyme A (acyl-CoA) to an acceptor alcohol, found in plants, yeast, filamentous fungi, and some bacteria.[4−6]

Acetate esters are produced by the condensation of ethanol or fusel alcohol with acetyl coenzyme A (acetyl-CoA),[7] which is derived from pyruvate or acetate by the action of alcohol-*O*-acetyltransferase (ATF).[8] ATF1 and ATF2 have been reported in *Saccharomyces cerevisiae* (Figure 1A).[9,10] ATF1 is crucial for the formation of acetate esters in *S. cerevisiae*,[8] which in turn attract insect vectors for dispersal.[11] In contrast, ATF2 is important for sterol detoxification because it facilitates the secretion of acetylated sterols into the culture medium.[12] ATF proteins contain a conserved domain, the HXXXD motif, which transfers an acetyl group from acetyl-CoA to alcohol.[13] The second paralogs of AATases, having an α/β hydrolase fold structure and the Ser−Asp/Glu−His catalytic triad,[14] include ethyl ester biosynthesis 1 (EEB1) and ethanol hexanoyl transferase 1 (EHT1).[15] The acyl chain derived from fatty acyl-CoA and ethanol are polymerized by the reaction of EEB1 and EHT1 in *S. cerevisiae*, generating MCFA-ethyl esters to eliminate the MCFAs that accumulate during fatty acid synthesis (Figure 1A).[14,16] Ethanol acetyltransferase 1 (EAT1), a recently discovered member of the yeast AATase family, produces ethyl acetate in mitochondria.[17] EAT1 is a key enzyme responsible for producing large amounts of ethyl acetate in several yeast species, including *Wickerhamomyces anomalus* and *Kluyveromyces marxianus*.[17,18]

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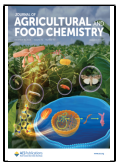

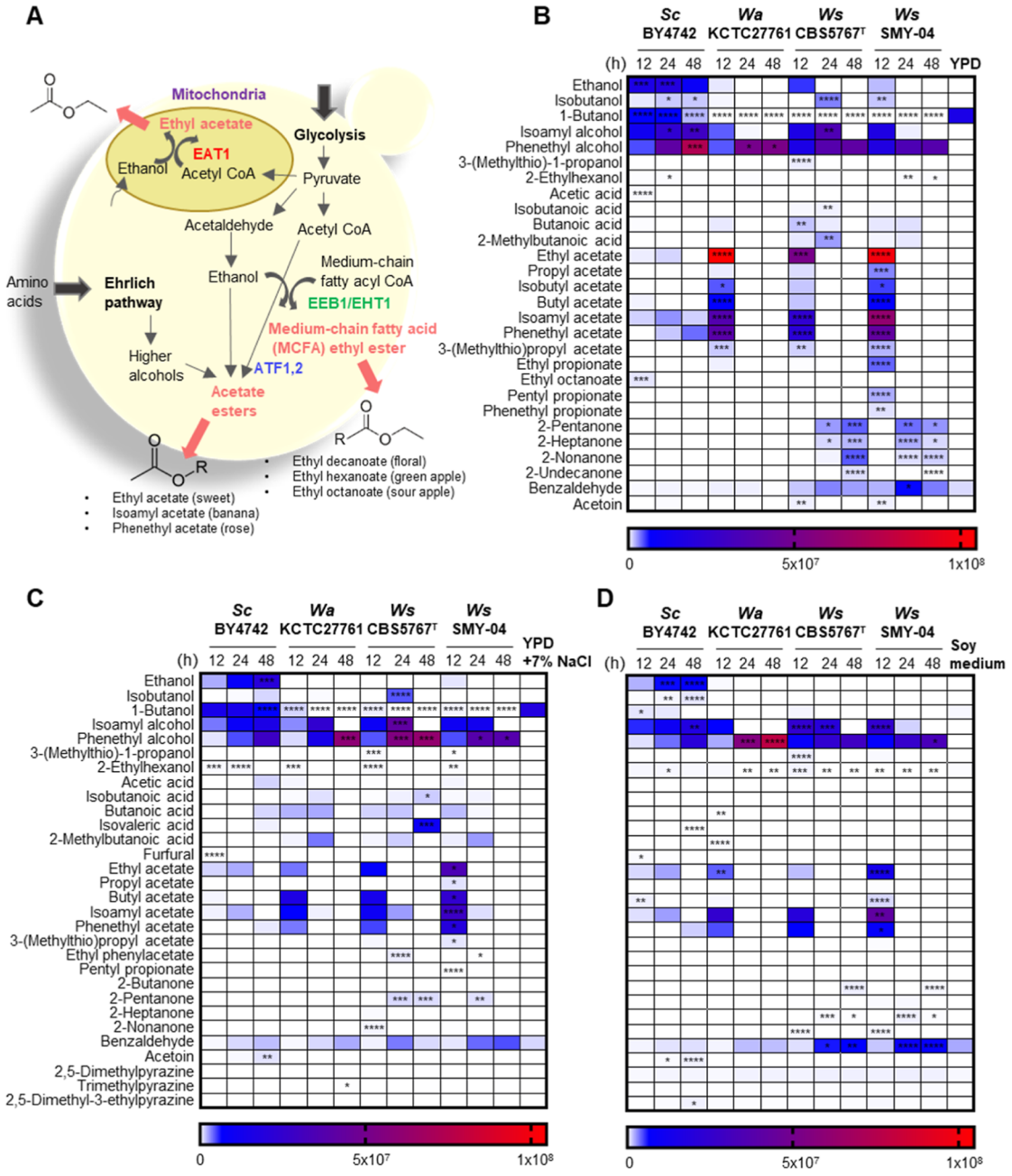

Figure 1. Volatile flavor profiles of *W. subpelliculosus*. (A) Biosynthesis pathways of acetate esters and MCFA ethyl esters in yeast. (B–D) Volatile flavor profiles of *S. cerevisiae* (Sc) BY4742, *W. anomalus* (Wa) KCTC27761, and *W. subpelliculosus* (Ws) CBS5767^T and SMY-04 strains cultivated in YPD, YPD with 7% NaCl, and soy fermentation mimic medium at 28 °C, respectively. The culture supernatants collected at 12, 24, and 48 h were analyzed by HS-SPME GC/MS. Each heatmap shows the relative peak area extracted from the culture supernatants compared to media alone (YPD, YPD with 7% NaCl, or soy fermentation mimic medium). GraphPad Prism (ver. 8.4) was used to generate the plots. *P < 0.05; **P < 0.01; ***P < 0.001; and ****P < 0.0001, between cell cultures and media without yeast inoculation (one-way ANOVA).

Wickerhamomyces subpelliculosus is a non-*Saccharomyces* yeast widely found in fruits, vegetables, flowers, and fermented foods, including soy sauce, cucumber pickles, and sausages, as a flavor producer.[19−21] In this study, we report the first genome sequence information on *W. subpelliculosus* from *de novo* whole-genome sequencing of a CBS5767^T strain isolated from pickled cucumber and an SMY-04 strain isolated from Korean traditional fermented soy sauce, ganjang. Based on the obtained genome sequence information, we identified *W. subpelliculosus* AATase genes encoding five ATF homologues, one EAT1 homologue, and one EHT1 homologue, which were characterized based on the *in vivo*/*in vitro* enzymatic activity and cellular localization analyses.

2. MATERIALS AND METHODS

2.1. Strains, Plasmids, and Cultivation Conditions. The yeast strains, plasmids, and primers used in this study are given in Tables S1, S2, and S3, respectively. The construction of yeast strains and expression vectors are described in Supporting Information. Yeasts were grown in YPD (1% yeast extract, 2% Bacto-peptone, and 2% glucose), soy fermentation mimic medium (1% yeast extract, 2% soy peptone, 0.5% glucose, and 7% NaCl), GB medium (glycerol-rich broth containing 0.2% yeast extract, 0.5% KNO_3, 0.1% citric acid,

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0.15% $(NH_4)_2SO_4$, 0.1% $MgSO_4$, 0.2% $CaCl_2$, 8% glycerol, and appropriate amounts of leucine and uracil), or synthetic complete medium (SC; 0.67% yeast nitrogen base without amino acids, 2% glucose, and 0.77 g/L drop-out amino acid mixture) at 28 °C. For yeast transformation, the LiAc/SS carrier DNA/PEG method was used.[22] The yeast transformants were cultured in SC-H medium (SC medium lacking histidine) or SC-H-L (SC medium lacking histidine and leucine).

2.2. Whole-Genome Sequencing and Annotation. A SMRTbell library was prepared for sequencing on a PacBio Sequel using the Sequel Binding Kit 2.1 (PacBio, Menlo Park, CA, USA), in accordance with the manufacturer's protocol. After quality control, the preprocessed long reads were assembled using the HGAP4 *de novo* assembly pipeline (ver. 4.0) with default settings to construct a draft whole-genome assembly.[23] Highly accurate sequences from Illumina HiSeq were employed for error correction using Pilon (ver. 1.21) based on the mapping outcomes of short reads against the assembled contigs. The polishing process was carried out with the combination of sequencing platforms and assembly tools reported in previous analyses.[24−27] The polished whole-genome assembly was subjected to gene prediction using MAKER (ver. 2.31.8), an *ab initio* gene prediction tool.[28] The predicted genes were functionally annotated using BLAST+ (version 2.6.0) and referring to the following databases: UniProt (ver. 201806), InterPro (ver. 69.0), Pfam (ver. 31.0), CDD (ver. 3.16), TIGRFAMs (ver. 15.0), and EggNOG (ver. 4.5.1).[29−35] Additional analyses were conducted to determine the repeat structures of the assembled contigs using RepeatMasker (ver. 4.1.2-p1, http://repeatmasker.org) and noncoding RNA regions, including rDNA and tRNA, using tRNAscan-SE (ver. 2.0.9) and Infernal (ver. 1.1.4).[36,37] The GC content of the assembled genome was determined using GCcalc.py (https://github.com/WenchaoLin/GCcalc) and Quast software (ver. 5.0.2).[38] Contigs, genes, repeat sequences, noncoding RNAs, and GC contents were integrated and visualized using Circos (ver. 0.69−8).[39]

2.3. Volatile Aroma Compound Analysis by Headspace Solid-Phase Microextraction Followed by Gas Chromatography/Mass Spectrometry. Yeast cells were cultivated for 12, 24, and 48 h in various media such as YPD, YPD with 7% NaCl, soy fermentation mimic medium, or SC-H at 28 °C and 220 rpm. After centrifugation, the cell-free supernatant was transferred to a 20 mL vial with a polytetrafluoroethylene/silicon septum (Agilent Technologies, Santa Clara, CA, USA). The samples were analyzed using the splitless mode of the HS-SPME GC/MS system (7820A/5977E MSD, Agilent Technologies) with a DB-wax column (50 × 200 μm × 0.2 μm; Agilent Technologies) at a flow rate of 1 mL/min (helium gas). The equilibrium process proceeded for 5 min at 50 °C. The volatile compounds were absorbed onto a 50/30 μm DVB/CAR/PDMS fiber for 30 min and then desorbed into the injection port for 2 min at 250 °C. The oven temperature was initially increased for 5 min from 40 °C to 150 °C at increments of 5 °C/min and to 200 °C at increments of 7 °C/min. The 200 °C temperature was maintained for 10 min. MS was performed in SCAN mode (m/z 33−200) with an ionization energy of 70 eV. Compounds were identified using the NIST Mass Spectral Search Program (ver. 11). The compounds were quantified in SCAN mode by using a total ion chromatogram on an arbitrary scale. Quantification of acetate esters was performed using HS-SPME GC/MS based on the standard curves of each acetate ester. The analysis was performed in duplicate.

2.4. Lipid Extraction and Detection of Long-Chain Bases by Thin-Layer Chromatography. Yeast cells were incubated in GB or SC-H medium for 4 days. After centrifugation, the cell pellets were suspended in methanol (0.1 g wet cake weight of cell pellet/1 mL methanol) and subjected to mild sonication for 30 min using Bransonic ultrasonic bath (Branson Ultrasonics, Brookfield, CT, USA), operating at 40 kHz. After centrifugation, the obtained supernatant was filtered and used as a cell surface sample. The lipid extracts in the obtained cell surface samples were separated by TLC using a developing solvent of chloroform/methanol/25% ammonia solution (85:15:10, v/v/v). The silica plate was dried and immersed in a detection solution (10% $CuSO_4$ in 8% H_3PO_4). After complete drying, the plate was baked at 180 °C overnight to develop the color for detecting LCB products.

2.5. Microsome Isolation and *In Vitro* Assay of LCB Acetylation Activity. Isolation of microsomes and *in vitro* acetylation assays on sphingoid bases were performed as previously described.[40] After 24 h cultivation in the SC-H medium, yeast cells were centrifuged (3390*g*, 10 min), washed with PBS, mixed with breaking buffer (0.125 mM sucrose, 0.5 mM EDTA, 125 mM KCl, 1 mM PMSF, and 1 mM DTT) and beads at a 1:1:1 ratio, vortexed for 1 min, and stabilized on ice for 1 min; this process was repeated 10 times. Subsequently, the samples were centrifuged to separate the pellet and the supernatant (1550*g*, 5 min, 4 °C). The obtained supernatant was ultracentrifuged (100,000*g*, 60 min, 4 °C), and the pellet was suspended in 200 μL of PBS as the final membrane fraction. The samples were stored in a 10% (v/v) glycerol and protease inhibitor mix [1 mM PMSF and protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA)] at −70 °C. The microsome fractions obtained were used in a reaction mixture [50 mM potassium phosphate buffer (pH 7.6), 1 mM LCB substrate in 5% sodium taurocholate, 0.75 mM acetyl-CoA, and 250 μg microsomal protein], which was then incubated at 37 °C for 90 min and terminated by adding a stop solution (1.6 mL of 1:1 mixture of chloroform/methanol and 0.6 mL of water). After complete drying, the samples were vortexed with methanol and centrifuged (1550*g*, 20 min). The obtained supernatant was dried, dissolved in methanol, filtered, and analyzed by TLC.

2.6. *In Vitro* Activity Analysis of AATase, Thioesterase, and Esterase and *In Vivo* Alcoholysis Analysis. All *in vitro* assays were performed by using yeast soluble cell lysates. *S. cerevisiae* cells were inoculated in 20 mL of SC-H medium at an initial OD_{600} of 0.5 and cultivated for 12 h at 28 °C. After centrifugation, cells were resuspended (at a total OD_{600} of 50) in 150 μL of lysis buffer [10 mM potassium phosphate (pH 7.5), 0.8 mM magnesium chloride, 5 mM DTT, 2 mM PMSF, and protease inhibitor] and disrupted using glass beads using a Precellys 24 tissue homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France). The yeast cell lysates were then centrifuged (1550*g*, 10 min, 4 °C), and the total protein concentration was determined using the Bradford method. *In vitro* AATase assay for the synthesis of MCFA-ethyl esters was conducted for 1 h at 30 °C in a mixture (200 μL) containing 200 mM KH_2PO_4 (pH 7.5), 0.5 M ethanol (EtOH), and 0.5 mM hexanoyl-CoA, octanoyl-CoA, and decanoyl-CoA, respectively, in the presence of 100 μg of cell lysate. The reaction was terminated by adding 5.5 μL of 3 M H_2SO_4. Hexanoyl-CoA, octanoyl-CoA, and decanoyl-CoA were purchased from Sigma-Aldrich. Ethyl hexanoate, ethyl octanoate, and ethyl decanoate synthase activities were measured by using HS-SPME GC/MS. *In vitro* thioesterase assay was performed in the same reaction mixture as the AATase assay but in the absence of EtOH. Esterase activity with *p*-nitrophenyl esters as substrates was determined based on the amount of *p*-nitrophenol released by esterase-catalyzed hydrolysis. Stock solutions of 5 mM *p*-nitrophenyl esters were prepared in methanol. The yeast cell lysates (total of 40 μg of soluble proteins) were incubated at 30 °C for 1 h with 50 μM *p*-nitrophenyl acetate, *p*-nitrophenyl hexanoate, *p*-nitrophenyl octanoate, or *p*-nitrophenyl decanoate in 50 mM sodium phosphate buffer, pH 7.4, and 150 mM NaCl in 96-well flat-bottom plates in a final volume of 150 μL. Liberation of *p*-nitrophenol was measured at various incubation times (0−60 min) at an absorbance of 400 nm. Normalized esterase activity was defined as the amount of total protein (mg) required to form 1 μmol of *p*-nitrophenol per min. For the *in vivo* alcoholysis assay, 5 mM butyl butyrate was added to the SC-H medium of the recombinant *S. cerevisiae* strains (OD_{600}: 0.5);[41] 5 mM butyric acid was added to the growth medium as a control. The cultures were incubated at 28 °C for 24 h with shaking at 220 rpm. After centrifugation at 16,600*g* for 5 min, the obtained culture supernatant (100 μL) was mixed with 10 mM acetone (100 μL) as an internal standard in a 20 mL gastight vial for HS-SPME GC/MS analysis. All data from the enzyme assays are the averages of biological triplicates, and the standard error of the mean is presented as an error bar in all figures.

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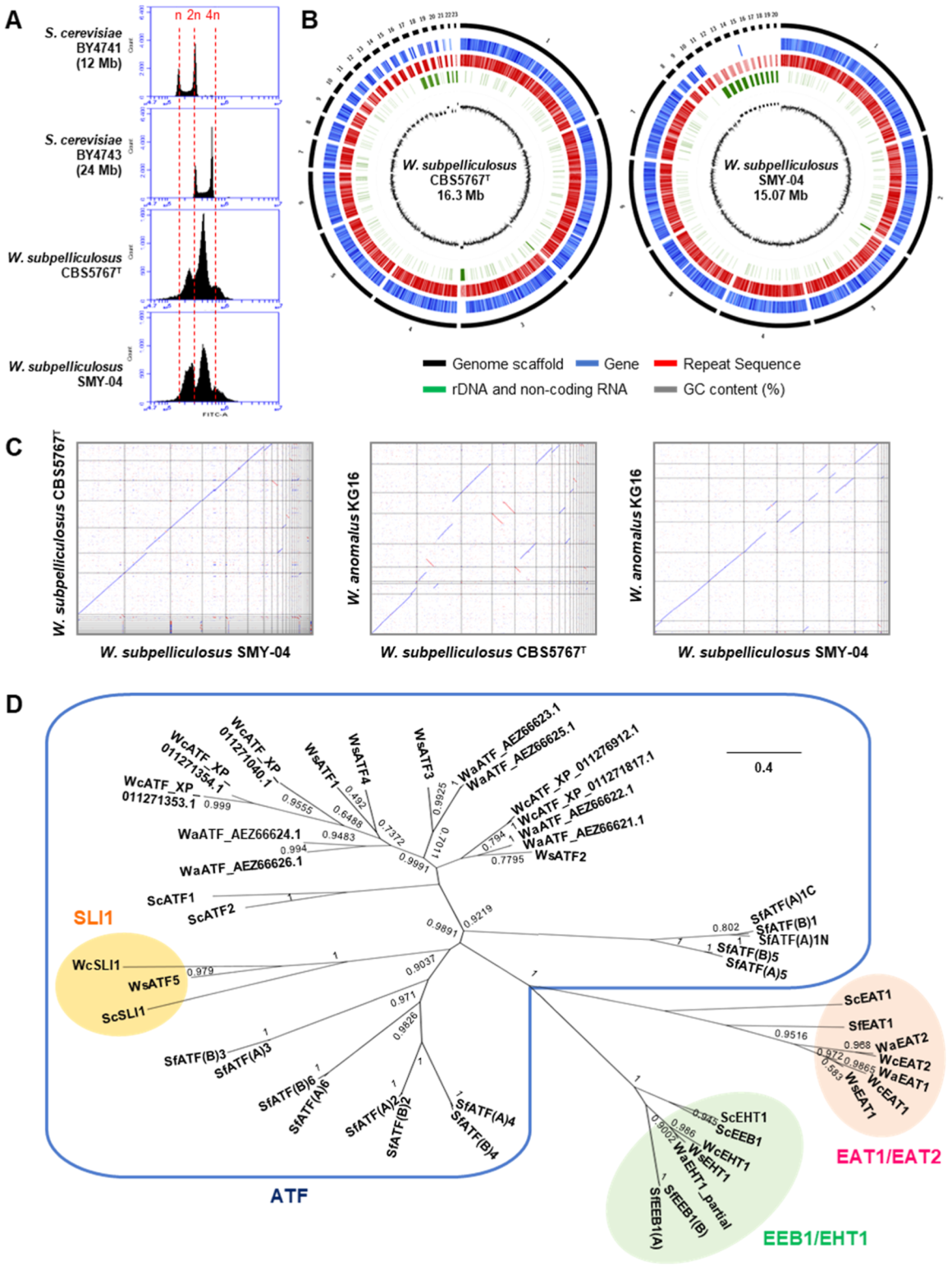

Figure 2. Analyses of ploidy, whole-genome sequencing, and putative AATase genes of *W. subpelliculosus*. (A) Flow cytometry analysis for ploidy determination. *S. cerevisiae* BY4741 (haploid) and *S. cerevisiae* BY4743 (diploid) were used as controls. The flow cytometry analysis using SYTOX green nucleic acid stain was carried out as described previously.[45] (B) Circos plot of genome scaffolds (black), protein annotation (blue), repeat annotation (red), noncoding RNA and rDNA (green), and GC contents (inner black). (C) Synteny analysis of *W. subpelliculosus* and *W. anomalus* KG16, an isolate from Korean traditional soy sauce. The synteny blocks were visualized using dot plots. (D) Phylogenetic analysis of AATase proteins in various yeast species. To construct the phylogenetic tree using Jones−Taylor−Thornton (JTT) model, MAFFT (ver. 7.475) was used as a multiple sequence alignment tool and RAxML-NG (ver. 1.0.3) as a tree construction tool. The bootstrap value was 1000, and bs-metric (tbe) was set. Bar, 0.4 substitutions per site. The NCBI numbers of AATase orthologs are listed in Table S10. Sc: *S. cerevisiae* S288C, Sf: *S. fibuligera* KJJ81, Wa: *W. anomalus* NRRL Y-366−8, Wc: *W. ciferrii* NRRL Y-1031 F-60−10, and Ws: *W. subpelliculosus* CBS5767^T.

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2.7. Statistical Analysis. Statistical differences between multiple groups were evaluated via one-way or two-way analysis of variance (ANOVA) based on the involvement of independent variables. Statistically significant data were presented with p-value < 0.05. These analyses were conducted in GraphPad Prism (ver. 8.4).

3. RESULTS

3.1. Growth Characteristics and Volatile Aroma Profiles of *W. subpelliculosus* Strains. *W. subpelliculosus* is phylogenetically located within a *Wickerhamomyces* subclade containing *W. anomalus*, a key flavor enhancer of the Chinese liquor baijiu, and *Wickerhamomyces ciferrii* (synonym: *Pichia ciferrii*), a natural producer of sphingolipid derivatives,[42−44] as indicated by the phylogenetic tree analysis based on the 18S rRNA sequences of various *Wickerhamomyces* species (Figure S1A). Considering that both *W. subpelliculosus* CBS5767^T and SMY-04 strains were isolated from salted fermented foods, such as cucumbers pickled in brine and soy sauce, we compared the growth of the*W. subpelliculosus* strains with those of*S. cerevisiae*and other nonconventional yeast species, including*W. anomalus* and the halotolerant*Debaryomyces hansenii*, under various osmotic stress conditions (Figure S1B).

In salt- and osmotic-stress and low-temperature (10 and 15 °C) environments, most nonconventional yeast species, including *D. hansenii* CBS767^T, *D. hansenii* KCTC27743, *W. anomalus* KCTC27761, *W. subpelliculosus* CBS5767^T, and *W. subpelliculosus* SMY-04, were more resistant than *S. cerevisiae* BY4742. Notably, the *W. subpelliculosus* SMY-04 strain showed slightly stronger resistance to salt and osmotic stresses than CBS5767^T, which is plausible, considering the fermentation environments from which these strains were isolated, i.e., Korean soy sauce generally fermented in high-salinity brine (12−20%) and pickled cucumber fermented in low-salinity brine (2−8%).[46,47] These results indicate that *W. subpelliculosus* strains can grow vigorously under several stress conditions, including salt, osmotic, temperature, and acid stresses, which are characteristic of fermented food environments.

To examine the flavor producibility of the *W. subpelliculosus* strains in the presence of NaCl and under the fermentation mimic condition, we compared the flavor profiles of the *W. subpelliculosus* to those of *S. cerevisiae* BY4742 and *W. anomalus* KCTC27761 cultivated in YPD, YPD supplemented with 7% NaCl, and soy fermentation mimic medium. In addition, since the availability of the substrates of the flavor producing enzymes changes as the cells grow, we decided to observe the flavor profile at 12, 24, and 48 h after cultivation. The volatile flavor profiles, analyzed by HS-SPME GC/MS, indicated that the flavor formation capacity of *W. subpelliculosus* CBS5767^T and SMY-04 was very similar to that of *W. anomalus* KCTC 27761 when cultured in YPD medium at 28 °C (Figure 1B and Table S4). Due to more vigorous growth of *W. anomalus* and *W. subpelliculosus* than *S. cerevisiae* (Figure S1B), the major flavor components were mostly detected at 12 h but disappeared at the stationary growth phase, probably because of the depletion of precursors required for the generation of flavor components (Figure 1B). Notably, the heatmap of the volatile compounds after 12 h of cultivation revealed that both *W. anomalus* and *W. subpelliculosus* produced substantially higher amounts of acetate esters, including ethyl acetate, propyl acetate, isobutyl acetate, butyl acetate, and 3-(methylthio)propyl acetate, which were barely detected in*S. cerevisiae*, suggesting that more diverse species of acetate esters are produced by*Wickerhamomyces* yeasts. In the*S. cerevisiae*-BY4742 strain, significantly higher amounts of ethanol and ethyl octanoate were detected than in*W. anomalus* and*W. subpelliculosus*. Some noticeable differences in the aroma profiles were also observed between *W. anomalus* and *W. subpelliculosus*: 2-methylbutanoic acid, pentyl propionate, and phenethyl propionate were detected only in*W. subpelliculosus*. Interestingly, after 24 h of cultivation, ketones, including 2-pentanone, 2-heptanone, 2-nonanone, and 2-undecanone, were detected only in*W. subpelliculosus*. In addition, *W. subpelliculosus* generated higher amounts of benzaldehyde than did *S. cerevisiae*or*W. anomalus* (Figure 1B). Particularly, the*W. subpelliculosus* SMY-04 strain isolated from soy sauce displayed outstanding flavor compound-generating features, in that, significant amounts of acetate esters and benzaldehyde were produced in the NaCl-supplemented YPD and soy fermentation mimic medium (Figure 1C,D, Tables S5, and S6). The quantitation of the three representative acetate esters, i.e., ethyl acetate, isoamyl acetate, and phenethyl acetate, which are the major acetate esters produced from*Wickerhamomyces* yeast species, revealed the superior production ability of*W. subpelliculosus* under the salt stress conditions compared to even*W. anomalus*, the well-known flavor producer under salty conditions (Table S7). After 12 h cultivation in YPD, ethyl acetate was produced ∼100 mg/L by*W. subpelliculosus* SMY-04, ∼50 mg/L by*W. subpelliculosus*CBS5767^T, and ∼120 mg/L by*W. anomalus* KCTC27761 (Table S7), which are below the threshold concentration (150 mg/L) as an off-flavor.[48]

We also analyzed and compared the growth of the *S. cerevisiae* KSD-YC strain used for Korean rice wine production with those of*W. subpelliculosus*and other yeast species under the stress conditions (Figure S1B).[49] The*S. cerevisiae* KSD-YC strain showed higher tolerance to 15% KCl, 2.5 M sorbitol, and low temperature than the*S. cerevisiae*BY4742 (haploid) and BY4743 (diploid) lab strains. Nevertheless,*S. cerevisiae*KSD-YC showed less tolerance than the*W. subpelliculosus*strains, particularly SMY-04, which exhibited the strongest tolerance to all of the stresses examined among the tested yeast species. This result further supports that *W. subpelliculosus*has a stronger tolerance to various stresses over many other yeast species. Additionally, we compared the flavor profile of the*S. cerevisiae*KSD-YC strain with that of*W. subpelliculosus*SMY-04 (Figure S2). Although the overall flavor profiles were similar between the KSD-YC production strain and the BY4742 lab strain,*S. cerevisiae*KSD-YC produced higher levels of acetoin, diacetyl, and isoamyl acetate than BY4742. The distinctive flavor profiles between*S. cerevisiae*and*W. subpelliculosus* were observed with both*S. cerevisiae* lab and production strains, supporting species-specific flavor profiles.

3.2. Genomic Features and *In Silico* Identification of AATase Genes in*W. subpelliculosus*. An initial investigation of the genomic features of *W. subpelliculosus* CBS5767^T and SMY-04 by flow cytometry indicated that the genomes of both strains were haploid (Figure 2A). By *de novo* whole-genome sequencing on PacBio Sequel and Illumina HiSeq 2500 systems, the CBS5767^T genome was assembled into 23 contigs with a total length of 16.3 Mb, and the SMY-04 genome was assembled into 20 contigs with a total size of 15.07 Mb (Figure 2B). Subsequent annotation predicted a nonredundant set of 7615 and 7027 protein-coding genes in the CBS5767^T and SMY-04 genomes, respectively (Table S8). Synteny analysis revealed that the gene orders and orientation are well conserved between the genomes of the two*W. subpelliculosus*

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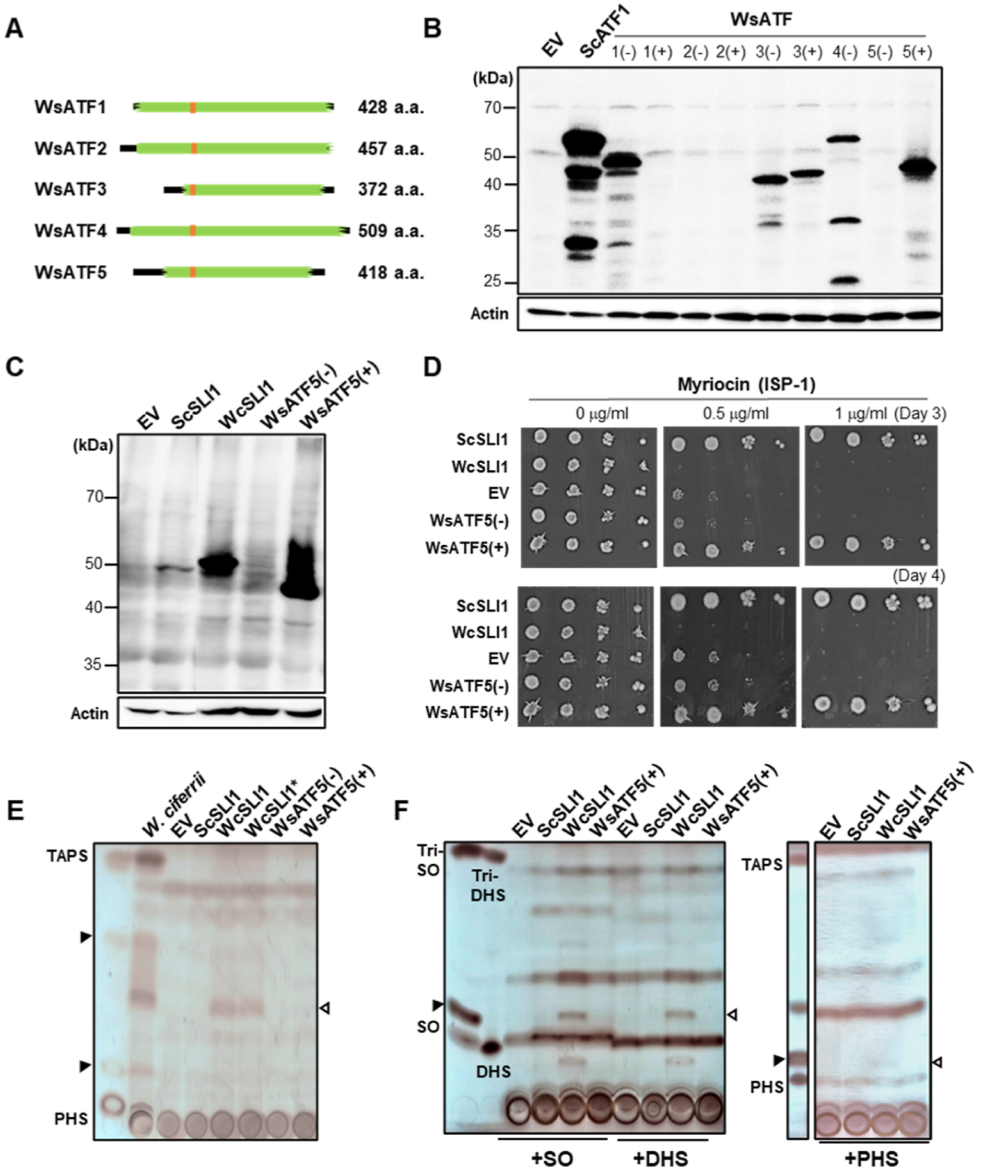

Figure 3. Expression and functional analysis of *W. subpelliculosus* ATF proteins in *S. cerevisiae*. (A) Domain analysis of WsATF proteins with AATase and conserved HXXXD domains. Structural analysis was conducted using the Pfam database (http://pfam.xfam.org). (B) Western blot analysis of WsATF proteins expressed in*S. cerevisiae*. Yeast cells harboring an empty vector YCpHT (EV) (Lane 1), the expression vectors YCpHT-ScATF1-6His (Lane 2), YCpHT-WsATF1(−)-6His (Lane 3), YCpHT-WsATF1(+)-6His (Lane 4), YCpHT-WsATF2(−)-6His (Lane 5), YCpHT-WsATF2(+)-6His (Lane 6), YCpHT-WsATF3(−)-6His (Lane 7), YCpHT-WsATF3(+)-6His (Lane 8), YCpHT-WsATF4(−)-6His (Lane 9), YCpHT-WsATF5(−)-6His (Lane 10), or YCpHT-WsATF5(+)-6His (Lane 11) were cultivated in SC-H for 24 h and subjected to western blot analysis. (±): (with/without) intron. (C), (D) Western blot and myriocin resistance analyses of the recombinant *S. cerevisiae* expressing SLI1 homologues, *S. cerevisiae* (ScSLI1), *W. ciferrii* (WcSLI1), and *W. subpelliculosus* (WsATF5). Spotting assay for growth was conducted on SC-H supplemented with myriocin. (E), (F) *In vivo* and *in vitro* acetylation activity analysis of SLI1 homologues in the recombinant *S. cerevisiae*. For the *in vivo* analysis, the cell surface samples from the recombinant *S. cerevisiae* strains cultured in YPD for 4 days were analyzed by TLC to detect acetylated LCBs. For the *in vitro* analysis, the microsomes from the recombinant *S. cerevisiae* strains were incubated with sphingosine (SO), dihydrosphingosine (DHS), or phytosphingosine (PHS). TAPS, tetraacetyl PHS; Tri-SO, triacetylsphingosine; and Tri-DHS, triacetyl-dihydrosphingosine. The arrow heads indicate acetylated LCB products, and the asterisk indicates WcSLI1 expression without His-tagging.

strains (Figure 2C, left). As suggested in the phylogenetic tree analysis (Figure S1A), the genome of*W. anomalus*KG16, an isolate from ganjang,[50] and those of *W. subpelliculosus* strains showed high synteny (Figure 2C, middle and right); this suggests recent divergence from a common ancestor.

Based on the *W. subpelliculosus* genome information, five homologues of ScATF1, coding for putative alcohol *O*-acetyltransferases with the AATase Pfam domain (PF07247), were identified and named as WsATF homologues (WsATF1, WsATF2, WsATF3, WsATF4, and WsATF5) in the order of amino acid sequence identity to ScATF1 (Figure S3A). All five putative homologues of ATF proteins from *W. subpelliculosus* CBS5767^T had a conserved HXXXD domain, which is commonly found in ATF orthologs from various yeast and fruit species (Figure S3B).[51,52] The identity of WsATF homologues to ScATF1 was generally low (16−28.9%). In addition, the *W. subpelliculosus* open reading frames (ORFs; WsEAT1 and WsEHT1), possessing an α/β hydrolase fold

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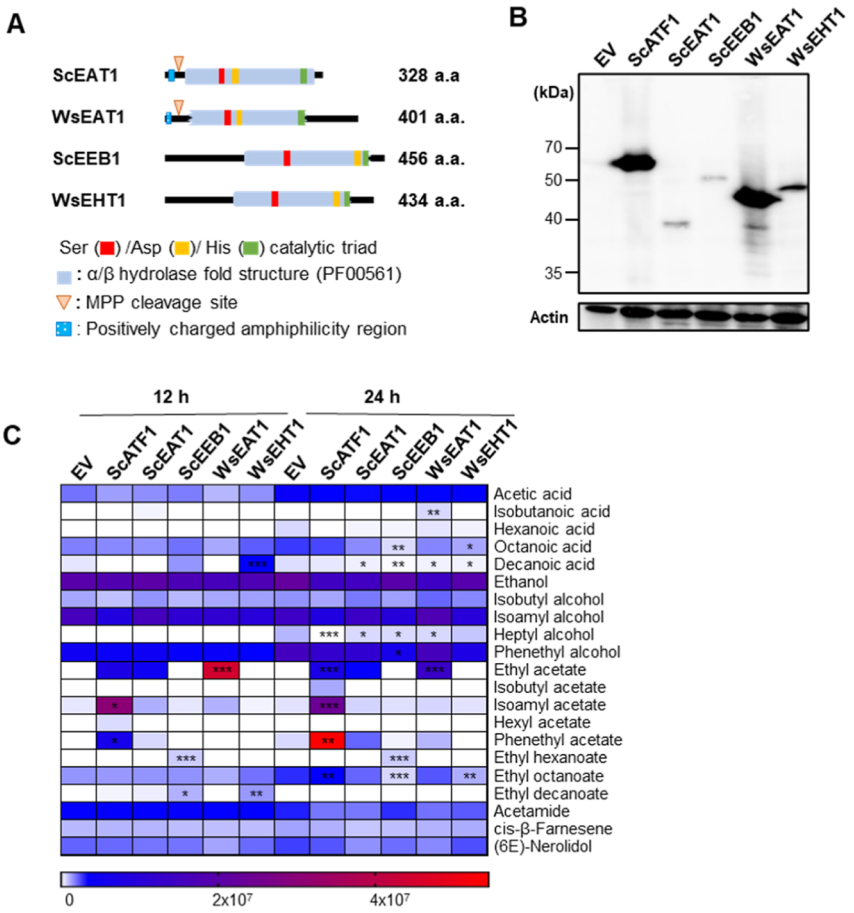

Figure 4. Expression and functional analysis of *W. subpelliculosus* EAT1 and EHT1 proteins in *S. cerevisiae*. (A) Predicted domain structure of yeast EAT1, EEB1, and EHT1 proteins. MPP, mitochondrial processing peptidase. (B) Western blot analysis of AATases expressed in *S. cerevisiae*. Each lane indicates cell lysates of the recombinant *S. cerevisiae* strains harboring an empty vector YCpHT (EV) (Lane 1), the expression vectors YCpHT-ScATF1-6His (Lane 2), YCpHT-ScEAT1-6His (Lane 3), YCpHT-ScEEB1-6His (Lane 4), YCpHT-WsEAT1-6His (Lane 5), and YCpHT-WsEHT1-6His (Lane 6). (C) Heatmap of volatile flavor compounds produced in the recombinant *S. cerevisiae* strains expressing ScATF1, ScEAT1, ScEEB1, WsEAT1, and WsEHT1 proteins, based on HS-SPME GC/MS analysis. Each culture supernatant was compared to that of the control *S. cerevisiae* strain (EV) using one-way ANOVA and Dunnett's multiple comparison test in GraphPad Prism (ver. 7.00). The observed differences were considered statistically significant at $P < 0.05$. *$P < 0.033$; **$P < 0.002$; and ***$P < 0.001$, between the control supernatant and each culture supernatant.

structure (Pfam domain: PF00561), were identified as homologues of EAT1 and EHT1 (Figure S4). The amino acid sequence identities of WsEAT1 and WsEHT1 were 25.4% and 48.7% compared to ScEAT1 and ScEHT1, respectively. Phylogenetic analysis was performed on AATase proteins from several yeast species, including *S. cerevisiae* S288C, *W. subpelliculosus* CBS5767^T, *W. anomalus* NRRL Y-366−8, *W. ciferrii* NRRL Y-1031, and *Saccharomycopsis fibuligera* KJJ81 (Figure 2D). The phylogenetic analysis showed that the AATase proteins diverged from a common ancestor into three major groups with distinctive structures and functions: ATF, EAT1/EAT2, and EEB1/EHT1 (Figure 2D). Interestingly, WsATF5 was more closely related to ScSLI1 and WcSLI1, which acetylate the sphingoid-base structure, than to other WsATF proteins with alcohol *O*-acetyltransferase activity for producing flavor ester compounds (Figure 2D). Except for WsATF5, the remaining WsATF proteins were grouped with alcohol *O*-acetyltransferases from *W. anomalus*, *W. ciferrii*, *S. fibuligera*, and *S. cerevisiae*.

3.3. *In Vivo* AATase Activity of *W. subpelliculosus* ATF Homologues. To clone the WsATF genes for expression in the heterologous host *S. cerevisiae*, the ORFs of each gene were amplified from *W. subpelliculosus* genomic DNA. Since introns were present in WsATF1, WsATF2, WsATF3, and WsATF5, they were removed through fusion PCR, generating both intron-containing (+) and intron-free (−) versions. Expression of these constructs in *S. cerevisiae* BY4742(*atf1*Δ*atf2*Δ) was confirmed by western blotting using an anti-His antibody (Figure 3A,B). The *S. cerevisiae* atf1Δatf2Δ double mutant was used as a host strain, in which acetate esters were hardly detectable (Figure S5A). Except for WsATF1(+), WsATF2(∓), and WsATF5(−), the proteins corresponding to WsATF1, WsATF3, WsATF4, and WsATF5 were detected, although at lower levels than that of *S. cerevisiae* ATF1 (Figure 3B). Despite the conservation of the functional domains in WsATF proteins, HS-SPME GC/MS did not detect an increase in acetate ester production (Figure S6), indicating that WsATFs may not be associated with the formation of aroma compounds.

The phylogenetic tree of AATase proteins from various yeast species (Figure 2D) suggests that WsATF5 may be associated with sphingoid-base *N*-/*O*-acetyltransferase activity. Previous studies showed that *S. cerevisiae* SLI1 (ScSLI1) has *N*-acetyltransferase activity toward myriocin, a sphingolipid

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biosynthesis inhibitor,[53] whereas *W. ciferrii* SLI1 (WcSLI1) has *N*/*O*-acetyltransferase activity toward long-chain bases (LCBs).[54] In the spotting assay of the recombinant *S. cerevisiae* strains expressing ScSLI1, WsATF5, and WcSLI1 in the presence of myriocin, the WsATF5-expressing strain exhibited resistance to myriocin (Figure 3C,D), comparable to the *S. cerevisiae* strain with additional ScSLI1 expression, indicating that WsATF5 is a functional homologue of ScSLI1. To further examine whether WsATF5 acetylates LCBs, including phytosphingosine (PHS), dihydrosphingosine (DHS), and sphingosine (SO), the secretory production of acetylated LCBs was examined by using TLC analysis of cell surface samples from the recombinant *S. cerevisiae* strains expressing ScSLI1, WsATF5, and WcSLI1 (Figure 3E). The secretion of LCBs, which are synthesized in the endoplasmic reticulum (ER), requires their acetylation by *N*/*O*-acetyltransferases.[54] PHS secretion by acetylation was detected only in the surface samples of *S. cerevisiae* expressing WcSLI1, while no acetylated PHS was detected in *S. cerevisiae* expressing ScSLI1 and WsATF5. This strongly indicates that *W. ciferrii* SLI1 can direct the secretion of LCBs at the cell surface of *S. cerevisiae*, whereas WsATF5 cannot acetylate PHS, as in the case of ScSLI1. To further analyze the acetylation activity toward the different sphingoid-base substrates, the microsomal fractions of the recombinant *S. cerevisiae* strains were allowed to react with SO, DHS, and PHS *in vitro* (Figure 3F). The results of *in vitro* LCB acetylation assays confirmed that WsATF5 has *N*-acetyltransferase activity toward myriocin but not LCBs, unlike WcSLI1, which showed acetylation activities toward SO, DHS, and PHS.

3.4. Enzymatic Activity of *W. subpelliculosus* EAT1 and EHT1 Homologues. Another group of AATase enzymes with α/β hydrolase fold structures, such as ScEAT1 and ScEEB1, is also involved in the production of volatile esters. From whole-genome sequencing data of *W. subpelliculosus* CBS5767^T, we identified the two ORFs encoding the EAT1 and EHT1 homologues (WsEAT1 and WsEHT1), containing an α/β hydrolase fold (PF00561) domain (Figure 4A). The conserved Ser−Asp/Glu−His catalytic triad was present in both WsEAT1 (Ser126, Asp150, and His300) and WsEHT1 (Ser247, Asp384, and His412), and the serine residue was located in a conserved domain, GXSXG (Figures 4A and S4). To examine the AATase activity associated with the generation of volatile esters, the *W. subpelliculosus* genes *WsEAT1* and *WsEHT1* and *S. cerevisiae* genes *ATF1*, *EAT1*, and *EEB1* were expressed in *S. cerevisiae* (Figure 4B). HS-SPME GC/MS of the culture supernatants obtained from the recombinant *S. cerevisiae* strains cultivated in SC-H medium (Figure 4C) showed that the WsEAT1-expressing *S. cerevisiae* strain produced markedly higher amounts of ethyl acetate after 12 h, which decreased slightly after 24 h. WsEAT1-expressing *S. cerevisiae* also produced isobutanoic acid and phenethyl acetate derived from fusel alcohol, even though the production levels were lower than those generated by ScATF1 expression at 24 h. The AATase activity of*W. subpelliculosus* EHT1 in the production of MCFA-ethyl esters was confirmed by the generation of a small amount of ethyl decanoate after 12 h, as observed with ScEEB1. The *in vivo* production of different acetate esters, ethyl acetate, and ethyl decanoate, respectively, as major products by the heterologous expression of WsEAT1 and WsEHT1 in *S. cerevisiae* supports their AATase activity with different substrate specificities.

W. anomalus EAT1 exhibits five distinct activities: AATase, esterase, thioesterase,[17] alcoholysis, and thiolysis (Table 1).[41] To examine the biochemical functions of*W. subpelliculosus*EAT1 and EHT1 in more detail, we performed an *in vitro* AATase activity assay for MCFA-ethyl ester generation using cell-free extracts of the recombinant*S. cerevisiae* strains expressing WsEAT1, WsEHT1, or ScEEB1. To exclude the intrinsic acyl-coenzyme A/ethanol *O*-acyltransferase activity of *S. cerevisiae*, a *Sceeb1*Δ deletion strain was used as the background strain (Figure S5B). In the reaction using yeast cell lysates with ethanol as the acyl acceptor and hexanoyl-CoA, octanoyl-CoA, and decanoyl-CoA as acyl donors, WsEHT1 showed AATase activity toward hexanoyl-CoA, generating ethyl hexanoate at levels comparable to the ScEEB1 treatment, whereas no significant amount of MCFA-ethyl esters was detected by WsEAT1 (Figure 5A, top). Surprisingly, the yeast cell lysates produced more MCFAs, such as hexanoic, octanoic, and decanoic acids, than the expected MCFA-ethyl esters. In particular, the WsEHT1 cell lysate generated significant amounts of the MCFAs with C8 and C10 lengths (Figure 5A, bottom). Given that MCFAs can be generated from esterase or thioesterase reactions, we performed thioesterase and esterase assays next. In the thioesterase reaction containing the yeast cell lysates and hexanoyl-CoA, octanoyl-CoA, or decanoyl-CoA as the only substrates, WsEHT1 showed remarkable thioesterase activity toward octanoyl-CoA and decanoyl-CoA (Figure 5B). In particular, WsEHT1 exhibited the highest thioesterase activity against octanoyl-CoA, consistent with *S. cerevisiae* EHT1 (Table 1),[14] which shows optimal activity toward octanoyl-CoA. These results strongly suggest that WsEHT1 exhibits stronger thioesterase activity than AATase activity *in vitro*. In addition, marginal levels of esterase activity were observed in all cell lysates of the recombinant *S. cerevisiae* strains expressing WsEAT1, WsEHT1, or ScEEB1 for *p*-nitrophenyl acetate, *p*-nitrophenyl decanoate, and *p*-nitrophenyl octanoate, respectively, compared to the control cell lysate from the strain carrying an empty vector (Figure 5C). Thus, although the statistical significance was not meaningful, the esterase activity of the AATase enzymes may also partially contribute to the production of MCFAs using the MCFA-ethyl esters, generated by the AATase activity, as substrates. Alcoholysis has been demonstrated *in vivo* in*S. cerevisiae* expressing several yeast EAT1 homologues cultivated with butyl butyrate supplementation.[41] In our study, ethyl butyrate was quantified as the product of the alcoholysis reaction between the supplemented butyl butyrate and endogenous ethanol in the engineered *S. cerevisiae* strains expressing WsEAT1, WsEHT1, ScEEB1, or ScEAT1 as a positive control. The *S. cerevisiae* cells expressing WsEAT1 produced much lower levels of ethyl butyrate, approximately one-quarter of that produced by ScEAT1-expressing *S. cerevisiae* cells, demonstrating its alcoholysis activity (Figure 5D). The lower production of ethyl butyrate might reflect the lower level of endogenous ethanol in *S. cerevisiae* cells expressing WsEAT1, due to the higher activity of WsEAT1 in converting ethanol to ethyl acetate than ScEAT1. Additionally, the different extents of the alcoholysis activity, which is a side activity of EAT1 proteins, might be ascribed to the low amino acid homology between *S. cerevisiae* and *W. subpelliculosus* EAT1 proteins.

3.5. Cellular Localization of the Functional *W. subpelliculosus* AATases: WsEAT1, WsEHT1, and WsATF5. *S. cerevisiae* and *S. fibuligera* ATF proteins were

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Table 1. Characteristics of AATases from Various Yeast Species[a]

	ATF1	ATF2	EAT1	EHT1	EEB1	SLI1
source	Sc, Sf	Sc	Sc, Wa, Km, Ws	Sc, Ws	Sc	Sc, Wc, Ws (WsATF5)
major product	acetate esters	acetate esters	ethyl acetate, other acetate esters	MCFA ethyl esters	MCFA ethyl esters	-*N*-acetylated myriocin (Sc, Ws), *N*-/*O*-acetylated long-chain base (Wc)
biological functions	attracting insects for dispersion	sterol metabolism	relieving acetyl-CoA accumulation	MCFA detoxification	MCFA detoxification	response to drug (Sc, Ws), Secretion of sphingolipids (Wc)
cellular location	ER, lipid droplets	ER, lipid droplets	mitochondria	ER, lipid droplets, mitochondria outer membrane	mitochondria	-ER (Sc), ER, lipid droplets (Ws)
protein family	AATase domain (PF07247)	AATase domain (PF07247)	α/β-hydrolase family (PF00561)	α/β-hydrolase family (PF00561)	α/β-hydrolase family (PF00561)	AATase domain (PF07247)
catalytic/conserved regions	HXXXD	HXXXD	Ser-Asp-His triad	Ser-Asp-His triad	Ser-Asp-His triad	HXXXD
side activities	thioesterase	unknown	thioesterase, esterase, alcoholysis	thioesterase, esterase	thioesterase, esterase	unknown
substrate specificity	fusel alcohols acetyl-CoA	fusel alcohols acetyl-CoA	ethanol, fusel alcohols acetyl-CoA	ethanol MCFA-CoA	ethanol MCFA-CoA	myriocin (Sc, Ws), sphingoid long-chain bases (Wc) acetyl-CoA

[a]The table was modified from Kruis *et al.* (2019)[3] with additional information for *S. fibuligera* ATF homologues,[56] *S. cerevisiae* SLI1,[53] *W. ciferrii* SLI1,[54] and *W. subpelliculosus* AATases (this study). Km, *K. marxianus*; Sc, *S. cerevisiae*; Sf, *S. fibuligera*; Wa, *W. anomalus*; Wc, *W. ciferrii* and Ws, *W. subpelliculosus*.

Figure 5. Enzyme activity assays of WsEAT1 and WsEHT1 using recombinant *S. cerevisiae* cell lysates. (A) *In vitro* alcohol acyltransferase assay for the generation of MCFA-ethyl esters and

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Figure 5. continued

(B) *in vitro* thioesterase assay. The levels of MCFA-ethyl esters (ethyl hexanoate, octanoate, and decanoate) and MCFAs (hexanoic, octanoic, and decanoic acids), produced by the EV control strain and ScEEB1, WsEAT1, and WsEHT1 overexpression strains, were measured by gas chromatography. (C) *In vitro* esterase assay. Esterase activity of each cell lysate toward *p*-nitrophenyl acetate, *p*-nitrophenyl hexanoate, *p*-nitrophenyl octanoate, and *p*-nitrophenyl decanoate was measured at an absorbance of 400 nm relative to a standard curve of *p*-nitrophenol. To calculate the normalized esterase activity, the activity (unit, U) was divided by the concentration of total proteins in cell lysate (mg). One U = 1 μmol min^{-1}. (D) *In vivo* alcoholysis assay using the recombinant *S. cerevisiae* expressing ScEAT1, ScEEB1, WsEAT1, or WsEHT1 grown for 24 h in the presence of 5 mM butyl butyrate. Data are presented as the mean and standard error of the mean from three biological replicate experiments. *P < 0.05; **P < 0.01; ***P < 0.001; and ****P < 0.0001, between activity in each cell lysate and that in the control cell lysate (two-way ANOVA).

localized to lipid droplets (LDs) or the ER due to the presence of N- and C-terminal dual amphipathic helix structures.[55,56] In *S. cerevisiae*, EHT1 localizes to the ER, outer mitochondrial membrane, and LDs. EEB1 and EAT1 have been traced to the yeast mitochondria.[57−61] We analyzed the cellular localization of WsEAT1, WsEHT1, and WsATF5, which were validated for the AATase activity in this study. WsEAT1 was predicted to contain a mitochondrial-targeting sequence with a mitochondrial processing peptidase cleavage site (Figure 4A). Fluorescence microscopic analysis of GFP fusion proteins showed that WsEAT1-yEGFP was located in the mitochondria, overlapping with the signals from the mitochondria marker MitoTracker dye, whereas the signals from the yEGFP control were dispersed throughout the cytosol (Figure 6A). In contrast, the localization patterns of yEGFP-fused WsEHT1 and WsATF5 overlapped with those of the ER tracker and ScErg6-DsRed, an LD marker, indicating localization to the ER and LDs (Figure 6B).

The cellular localization of EAT1 has been linked to its biological functions. Bulk ethyl acetate production is suggested to be associated with suboptimal growth conditions under which excess acetyl-CoA accumulates in yeast mitochondria. ATP citrate lyase, which converts citrate to acetyl-CoA and oxaloacetate at the expense of one ATP molecule, facilitates the transport of acetyl-CoA into the cytosol.[58] This reaction is typically observed in oleaginous yeasts such as *Yarrowia lipolytica* or *Rhodosporidium toruloides*.[62,63] With this enzyme, transport of acetyl-CoA from the mitochondria to the cytosol is feasible. When we performed a BLAST analysis of the *W. subpelliculosus* CBS5767^T genome using the sequence of ATP citrate lyase from *Y. lipolytica* CLIB122 (NCBI reference sequence XP_503231) as a query, we were unable to identify an ATP citrate lyase homologue. We hypothesized that acetyl-CoA accumulates within the mitochondria of *W. subpelliculosus* lacking ATP citrate lyase. Thus, EAT1-mediated ethyl acetate synthesis may reduce the accumulation of excess acetyl-CoA by converting acetyl-CoA to ethyl acetate in this yeast.

4. DISCUSSION

Various fermented food products have been produced with the traditional yeast *S. cerevisiae*. Recently, there has been a growing interest in nonconventional yeast strains with distinctive flavor profiles and strong stress tolerance that can expand diversity and complexity in fermented products, including bread[64,65] and alcoholic beverages.[66,67] *W. subpelliculosus* is considered an alternative baker's yeast due to its leavening function and higher stress tolerance, with a broader aroma profile than *S. cerevisiae*.[21] Recently, *W. subpelliculosus* strains with improved fermentative capacity, leavening ability, stress tolerance, and bread quality have been developed using evolutionary engineering.[68] Fruit-associated *W. subpelliculosus* is also a candidate of broad-spectrum biocontrol agents with an especially broad range and intensity of antagonism against various molds, bacteria, and yeast species.[69] Furthermore, this yeast also has probiotic potential in that it can modulate the human intestinal microbiota, as evidenced by increased

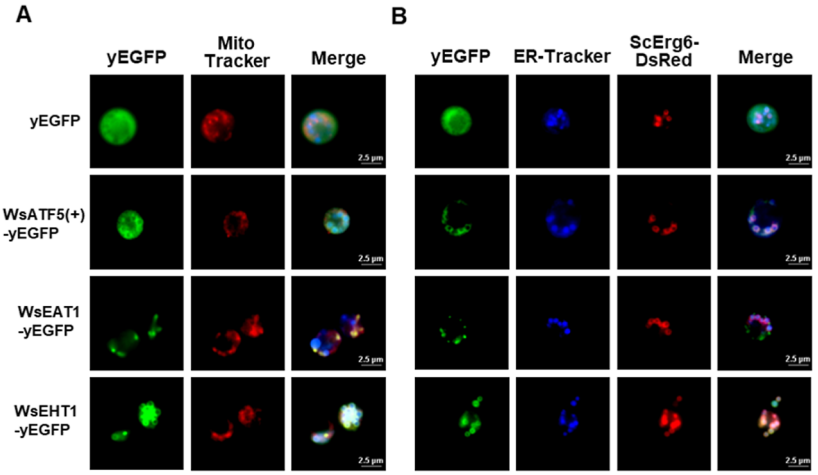

Figure 6. Fluorescent microscopic analysis of intracellular GFP-fused WsAATase protein localization in *S. cerevisiae*. The signals of yEGFP, WsATF5(+)-yEGFP, WsEAT1-yEGFP, and WsEHT1-yEGFP were observed with the mitochondria marker, MitoTracker dye (A) and with the ER-Tracker dye and the LD marker, ScErg6-DsRed (B). Yeast cells expressing AATase-yEGFP were cultivated in SC-H and the strains coexpressing AATase-yEGFP and ScERG6-DsRed were incubated in SC-H-L medium for 24 h. The yeast cells were subsequently cultured for 48 h in oleic acid medium, harvested, fixed, and treated with the mitochondrial staining reagent (MitoTracker Red CMXRos, Thermo Fisher Scientific, Waltham, MA, USA) and the ER staining reagent (ER-Tracker Blue-White DPX, Thermo Fisher Scientific), as described previously.[56] The fluorescence signals of yEGFP, MitoTracker/DsRed, and ER-Tracker are shown in green, red, and blue, respectively. Scale bar = 2.5 μm.

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beneficial microbes in treated fecal batches.[70] The present study reports the first whole-genome information on the fermented food isolates *W. subpelliculosus* CBS5767^T and SMY-04, which produce more diverse species of acetate esters compared to *S. cerevisiae* (Figures 1 and 2). The whole-genome-based information on *W. subpelliculosus* AATase proteins, including ATF, EAT1, and EHT1 homologues, was described and used to analyze their enzymatic activity associated with volatile ester generation (Table 1).

Although all five orthologous WsATFs contained the AATase Pfam domain (PF07247), we were unable to validate their function as alcohol-*O*-acetyltransferases in the generation of acetate esters in heterologous expression analysis. Nevertheless, the results indicated that *W. subpelliculosus* ATF5 is a functional homologue of *S. cerevisiae* SLI1, which has AATase activity different from that of WcSLI1, which serves as a sphingoid base *N*/*O*-acetyltransferase (Figure 3E,F). A few studies have validated the function of ATF proteins from non-*Saccharomyces* yeasts, such as *Kluyveromyces lactis*,[51] *W. anomalus*,[17] and *S. fibuligera*.[56] In the previous analysis of the diploid genome of *S. fibuligera* KJJ81, an isolate from a wheat-based nuruk in Korea, a total of 12 ATF orthologs (SfATFs) were identified as putative AATases. However, only three *S. fibuligera* ORFs, SfAtf(A)2p, SfAtf(B)2p, and SfAtf(B)6p, have been validated as functional AATases for acetate ester formation in the heterologous host *S. cerevisiae*.[56] Intriguingly, the endogenous expression of the *S. fibuligera ATF* genes without AATase activity at the transcript level was quite low.[56] Consistently, the expression levels of the *WsATF* genes, except for *ATF3* of CBS5767^T, were barely detected, even though the production of acetate esters was observed in both *W. subpelliculosus* strains cultivated under the same condition for qRT-PCR analysis (Figure S7). These results strongly suggest that the loss of AATase activity occurred frequently in ATF homologues during evolution of yeast species, reflecting their high divergence among yeast ATF homologues (Figures 2D and S3).[56,71] This notion is supported by a previous finding on *Zygosaccharomyces* and *Torulaspora* yeast species, which can produce acetate esters seemingly without ATF homologues, suggesting that ancestral ATF may have been lost independently several times during evolution.[72] Future studies should investigate other genes involved in ester synthesis in non-*Saccharomyces* yeasts expressing nonfunctional or lacking *ATF* genes. Under certain conditions, the reverse activity of esterases affects ester formation. For example, the balance between AATase and IAH1 (isoamyl acetate-hydrolyzing esterase) is important for isoamyl acetate accumulation.[73] *W. subpelliculosus* also has several homologous genes encoding a IAH1 homologue along with several esterases and thioesterases in its genome, which remains as an extensive field to be investigated.

In contrast to WsATFs, WsEAT1 can generate a large amount of ethyl acetate as a functional AATase, and WsEHT1 participates in MCFA-ethyl ester production (Figure 4). We also detected the higher endogenous expression of EAT1 at the transcript level in SMY-04 than in CBS5767^T, which is consistent with higher production of ethyl acetate of the strain than that of CBS5767^T (Figures 1B and S7). The expression of EHT1 and SLI1 was also detectable in both strains, although the expression level is lower than that of EAT1 (Figure S7). Previously, EAT1-like AATases with the ability to produce ethyl acetate were found to optionally possess a glutamic acid-arginine-proline (ERP), glutamic acid-asparagine-proline (ENP), or glutamic acid-methionine-proline (EMP) fragment as the fifth, sixth, and seventh amino acids, respectively, from the histidine of the catalytic triad on the *C*-terminus of the polypeptide.[74] Amino acid sequence alignment analysis of EAT1/EAT2 homologues from *W. subpelliculosus*, *S. cerevisiae*, *K. marxianus*, *K. lactis*, *W. anomalus*, and *W. ciferrii* revealed that the ERP/ENP/EMP conserved fragment was detected in all EAT1 homologues but not in EAT2 homologues from *W. anomalus* and *W. ciferrii* (Figure S4A), indicating that the ERP/ENP/EMP fragment functioned in generating ethyl acetate. In addition to the biotechnological importance of ethyl acetate as a flavor compound and the most abundant ester in wine and beer,[2] it is also used as a chemical solvent in the synthesis of biodiesels, paints, adhesives, herbicides, and resins.[17]

To produce esters, nucleophilic serine attacks the acyl-CoA substrate and forms an acyl-enzyme intermediate.[75] The acyl moiety of the intermediate combines with either ethanol or water, generating esters (AATases) or leading to acyl-CoA hydrolysis (thioesterase), respectively.[17,75] Ester hydrolysis (esterase) follows the same mechanism, except that an ester becomes the acyl donor and water is used to produce the acyl-enzyme intermediate.[75] As a side activity, ATF1, EEB1, EHT1, and EAT1 function as esterase and thioesterase by hydrolyzing volatile esters and acyl-CoA, respectively, *in vitro* (Table 1).[14,15,17] Our *in vitro* enzymatic activity assay revealed that WsEHT1 exhibited much higher thioesterase than AATase activity, particularly against octanoyl-CoA (Figure 5A,B). Acyl-CoA hydrolysis by thioesterase activity reduces ester production in the host cells. Engineering WsEHT1 to favor AATase activity over hydrolysis could be an effective means of increasing ester yields in synthetic cell factories. Alcoholysis is a transesterase reaction in which the alcohol moiety of an ester is replaced by another alcohol and likely affects ester generation and distribution both quantitatively and qualitatively in food and beverage production. We detected a moderate level of alcoholysis side activity by WsEAT1 expressed in *S. cerevisiae*, which sheds new light on the variety and production of short-chain esters in food and beverage products, highlighting a potential avenue for sustainably producing short-chain esters.

Besides ethyl esters, *W. subpelliculosus* produced remarkable amounts of phenethyl acetate, phenethyl alcohol, and benzaldehyde when cultivated in YPD, NaCl-supplemented YPD, or soy fermentation mimic medium (Figure 1B−D). A previous study proposed the biosynthetic pathway of benzenoid compounds, such as benzaldehyde and benzyl alcohol, through the mandelate pathway, with phenylpyruvate as an intermediate, in the wine yeast *Hanseniaspora vineae*.[76] The increased formation of phenethyl acetate, phenethyl alcohol, and benzyl alcohol in *H. vineae* was likely due to the duplicated *ARO8*, *ARO9*, and *ARO10* genes involved in the Ehrlich and mandelate pathways.[71] In the *W. subpelliculosus* genome, we identified five *ARO9* copies and three *ARO10* copies, which may have promoted benzaldehyde production from phenylalanine. Simultaneously, three *ADH3* copies, four *ADH4* copies, two *ADH7* copies, and ten *AAD16* copies in *W. subpelliculosus* may enable efficient conversion from phenylacetaldehyde to phenethyl alcohol. Through further validation of these flavor-associated genes and their functions, together with understanding of the regulatory mechanism on the flavor biosynthesis based on the combined analyses of transcriptomics and metabolomics, *W. subpelliculosus* would be

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exploited as a promising flavor enhancer in the field of bread and high-salted food fermentations.

In conclusion, we showed that the *W. subpelliculosus* isolates from salty fermented foods generate diverse species of volatile esters, with distinct acetate ester profiles generated under soy fermentation-mimicking condition, supporting the potential of *W. subpelliculosus* as a flavor producer in fermented food, as previously suggested in the fermentation of sausages and cherry puree.[19,70] This is the first study to report the genome-wide identification and systematic characterization of *W. subpelliculosus* AATase proteins via several biochemical assays of enzymatic activities, demonstrating their biotechnological potential for volatile ester production. However, to gain a more intensive understanding of the fermentation features of this yeast in various fermented foods, such as bread, salted vegetables, and soy sauce, it is necessary to apply this yeast in practical food fermentation and to evaluate the fermentation traits including flavor. The genomics-based functional study of aroma-related genes in the present study provides a valuable reference of multiomics analyses for the development of yeast strains to produce key flavor components in various food industries.

■ ASSOCIATED CONTENT

Data Availability Statement

The whole genome data of *W. subpelliculosus* SMY-04 and *W. subpelliculosus* CBS5767^T have been deposited in the NCBI database under accession numbers JAIZYX0000000 and JAIZYZ0000000, respectively. The sequences of *W. subpelliculosus* AATase genes have been deposited in NCBI under accession numbers OP485094 for WsATF1, OP485095 for WsATF2, OP485096 for WsATF3, OP485097 for WsATF4, OP485098 for WsATF5, OP485099 for WsEAT1, and OP485100 for WsEHT1.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.4c08103.

Description for construction of *S. cerevisiae* null mutant strains lacking *ATF1*, *ATF2*, and *EEB1*; description for construction of *S. cerevisiae* vectors for AATase and AATase-GFP fusion expression; yeast strains used in this study; plasmids used in this study; primers used in this study; relative peak area of the volatile compounds produced from the yeast strains cultivated in YPD; relative peak area of the volatile compounds produced from the yeast strains cultivated in YPD+7% NaCl; relative peak area of the volatile compounds produced from the yeast strains cultivated in soy fermentation mimic medium; quantification of acetate esters from the yeast strains by HS-SPME CG/MS analysis; whole-genome sequencing and annotation of *W. subpelliculosus* CBS5767^T and SMY-04; GenBank accession numbers of 18S rRNA from the genus *Wickerhamomyces* used in the phylogenetic tree analysis; AATase orthologs from various yeast species used in the phylogenetics analysis; phylogenetic and growth analyses of *W. subpelliculosus*; volatile flavor profiles of *S. cerevisiae* KSD-YC and *W. subpelliculosus* SMY-04 strains; sequence identity and multiple sequence alignment of WsATF proteins to yeast ATF homologues; multiple sequence alignment of EAT1, EEB1, and EHT1 from various yeast species; HS-SPME GC/MS profiles of volatile compounds produced by *S. cerevisiae* BY4742 wild-type, *atf1*Δ*atf2*Δ, and *eeb1*Δ mutant strains; HS-SPME GC/MS profiles of volatile compounds produced by the recombinant *S. cerevisiae* strains expressing the *WsATF* genes; and quantitative real-time PCR analysis of the AATase genes in the *W. subpelliculosus* CBS5767^T and SMY-04 strains (PDF)

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Notes

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