



Transcriptomic analysis of the metabolic characteristics of *Aspergillus oryzae* during the fermentation of doenjang-meju, a traditional Korean fermented soybean brick

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ABSTRACT

To investigate the fermentative traits of *Aspergillus oryzae* in doenjang-meju, a traditional Korean fermented soybean brick, we prepared doenjang-meju bricks inoculated with both *A. oryzae* and *Bacillus velezensis*—recognized as crucial microbes in doenjang-meju fermentation, and then the transcriptomes of *A. oryzae* were analyzed throughout the entire fermentation period. The transcriptomic analysis of carbohydrate-active enzyme (CAZy) genes revealed that *A. oryzae* exhibited robust expression of CAZy genes involved in the decomposition of various carbohydrates, particularly cellulose, starch, and hemicellulose, indicating the active breakdown of soybean carbohydrates by *A. oryzae* during fermentation. Furthermore, KEGG analysis, metabolic pathway reconstruction, and their transcriptomic analysis provided a comprehensive overview of the decomposition patterns of various carbohydrates, lipids, and proteins and the metabolic features of various carbon compounds during fermentation. Additionally, the transcriptomic analysis of biosynthetic gene clusters indicated the potential production of various secondary metabolites by *A. oryzae* during fermentation. Genes related to carbohydrate and carbon compound metabolisms and secondary metabolite biosynthesis displayed unique and different transcriptional expression patterns depending on the specific gene and fermentation stage. Overall, our findings provide comprehensive insights into the metabolic characteristics of *A. oryzae*, thus contributing to our understanding of soybean fermentation.

1. Introduction

In Korea, the traditional fermented soybean brick, known as doenjang-meju, plays a crucial role as a primary raw material in the traditional production of soybean products, namely doenjang (soybean paste) and ganjang (soy sauce) (Han et al., 2020, 2021). Traditional Korean doenjang-meju is crafted through the natural fermentation of steamed soybeans for 1–2 months, without the incorporation of starter cultures. This process leads to the proliferation of diverse microorganisms that originate from the surrounding environment, including *Aspergillus*, *Penicillium*, *Mucor*, *Rhizopus*, *Cladosporium*, *Bacillus*, *Enterococcus*, *Leuconostoc*, and *Pediococcus* (Heo et al., 2023; Jung et al., 2014; Kim et al., 2011, 2013, 2022; Ryu et al., 2021; Song et al., 2020). Nevertheless, numerous studies have consistently identified *Aspergillus* and *Bacillus* as the dominant and key fungi and bacteria, respectively, exerting a significant influence on the fermentation process of doenjang-meju (Gil et al., 2022; Hong et al., 2015; Kim et al., 2011,

2013, 2022).

Understanding the fermentative metabolic traits of key microbes responsible for traditional food fermentation is essential for advancing our knowledge of the fermentation process. To this end, extensive studies on the fermentative metabolism of *Bacillus* strains have been conducted, employing various approaches including genomic and transcriptomic analyses (Chun et al., 2019; Han et al., 2023; Li, Zheng, et al., 2023; Park et al., 2023). Some metabolic aspects of *Aspergillus oryzae*, recognized as a prominent species in koji and soybean fermentation, have also been explored in previous studies (Zhang et al., 2023; Zhao et al., 2015; Zhong et al., 2018). However, these studies have primarily focused on specific targets such as glycolytic (Zhong et al., 2018) and proteolytic enzymes (Zhang et al., 2023; Zhong et al., 2018), or have involved comparative analyses of transcriptional expressions between strains (Zhao et al., 2015; Zhong et al., 2018), without providing a comprehensive understanding of the fermentative metabolic features of *A. oryzae* throughout fermentation periods. Notably, the

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genomic and fermentative characteristics of *A. oryzae* during doenjang-meju fermentation have not been explored, despite its dominant presence in doenjang-meju.

The metabolic characteristics of microbes in food fermentation have mainly been studied by inoculating single microbes (Xiong et al., 2014; Park & Kim, 2020), even though it is well known that their metabolic characteristics differ significantly when they are present together due to metabolic interactions compared to when they are alone (Devanthi et al., 2018; Yao et al., 2019). Therefore, we prepared doenjang meju bricks inoculated with both *A. oryzae* and *B. velezensis*, which are recognized as common and key microbes during doenjang-meju fermentation (Gil et al., 2022; Kim et al., 2022), to more accurately understand their metabolic characteristics during fermentation. Given that previous studies have extensively characterized the fermentation metabolic features of *B. velezensis* using various approaches, including genomic and transcriptomic analyses (Chen et al., 2023; Chun et al., 2019; Han et al., 2023), our study focused on analyzing the fermentative metabolic features of *A. oryzae*. To achieve this, we conducted transcriptomic analysis throughout the entire doenjang-meju fermentation process to comprehensively investigate the fermentative metabolic features of *A. oryzae*.

2. Materials and methods

2.1. Preparation of doenjang-meju bricks, sampling, and quantification of bacteria and fungi

To investigate the metabolic traits of *A. oryzae* during doenjang-meju fermentation, we prepared doenjang-meju bricks inoculated with *A. oryzae* MA1 and *Bacillus velezensis* MB1, isolated from doenjang-meju (Kim et al., 2022) as described in a previous study (Han et al., 2023). Briefly, 30 kg of dried yellow soybeans (*Glycine max* (L.) Merr.) were soaked overnight in tap water at room temperature, then autoclaved at 121 °C for 15 min. After cooling to approximately 35 °C, *A. oryzae* MA1 and *B. velezensis* MB1 were inoculated into the soybeans at concentrations of 10^3 spores/g-soybeans and 10^6 cells/g-soybeans, respectively, using the method described by Han et al. (2023). The inoculated soybeans were thoroughly blended, mashed, and shaped into bricks measuring approximately $22 \times 14 \times 9$ cm, yielding a total of 15 doenjang-meju bricks, each weighing approximately 3 kg. These bricks were maintained at 25–30 °C for 45 days. Sampling was performed at specific time intervals (5, 10, 20, 30, and 45 days), with three doenjang-meju bricks being collected at each time point. These three bricks were then mechanically ground using a blender (Hanil, South Korea) and combined for total RNA extraction.

The abundances of bacteria and fungi in the combined doenjang-meju were assessed as described previously (Jung et al., 2014). Briefly, 1 g of meju samples was resuspended and serially diluted in phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 , 2 mM KH_2PO_4 , pH 7.2). The diluted samples were then spread on trypticase soy agar (TSA; MCell, South Korea) and potato-dextrose agar (PDA; MCell) for the quantification of bacteria and fungi, respectively. The agar plates were incubated at 30 °C for 3 days, and the numbers of bacteria and fungi in doenjang-meju were calculated as colony forming unit (CFU) per gram. The process of viable cell counting was performed in triplicate for each sample.

2.2. Total RNA extraction from meju samples, sequencing, and processing

Total RNA extraction from the combined doenjang-meju samples was conducted using a formamide/EDTA method as described by Lee et al. (2019). Briefly, 0.2 g of doenjang-meju samples was mixed with 100 μL of RNase-free distilled water (DW; Invitrogen, USA), 500 μL of formamide, and 20 μL of 0.5 M EDTA (pH 8.0) in a 2 mL Lysing Matrix E tube (MP Biomedicals, USA). The mixtures were vigorously homogenized for 40 s at 6000 rpm using a FastPrep-24™ Classic bead beating

grinder (MP Biomedicals) and subsequently centrifuged for 10 min at 4 °C ($16,100 \times g$). The resulting supernatants were carefully transferred to new 2 mL tubes. An equal volume of RNase-free DW and two volumes of phenol/chloroform (5:1; Sigma-Aldrich, USA) were then added to the supernatants, after which the samples were thoroughly mixed by inversion. After centrifugation for 10 min, the upper aqueous phases were carefully transferred to new 2 mL tubes. Total RNA was then precipitated by adding an equal volume of cold isopropanol, followed by centrifugation. The resulting RNA precipitates were washed with 1 mL of 70% ethanol and subsequently dissolved in 100 μL of RNase-free DW for RNA sequencing.

The sequencing and processing of total RNA were performed according to previously described procedures (Chun et al., 2021). In brief, rRNA depletion and subsequent cDNA library construction were carried out using the TruSeq total RNA sample prep kit (Illumina, USA). The resulting cDNA libraries were sequenced on the Illumina HiSeq platform ($\times 101$ bp) at Macrogen (Korea). To ensure data quality, the sequencing reads were trimmed, and those shorter than 30 nucleotides were discarded using the Sickle adaptive trimming tool (Joshi & Fass, 2011). Using the BWA-MEM program (Li, 2013), sequencing reads corresponding to structural rRNA and tRNA were removed through sequence comparisons against the rRNA and tRNA genes of fungi and bacteria, employing a cutoff E value of less than 0.001. Afterward, the remaining high-quality sequencing reads were aligned to the genomes of *A. oryzae* MA1 using the BWA-MEM program with stringent criteria for best-match alignment, requiring a minimum identity of 90% and a minimum alignment length of 20 base pairs, obtaining only the mRNA sequencing reads of *A. oryzae* MA1 from the total sequencing reads.

2.3. Transcriptomic analysis of carbohydrate active enzyme (CAZy) genes of *A. oryzae* MA1 during fermentation

Coding sequences (CDS) within the genome of *A. oryzae* MA1 were identified using the AUGUSTUS software (Hoff & Stanke, 2013). The mRNA sequencing reads of *A. oryzae* MA1, processed as described above, were then best-mapped to these CDS using the BWA-MEM program. The transcriptional expression levels of the CDS of *A. oryzae* MA1 during fermentation were quantified as read counts per kilobase of each coding sequence per million mapped reads (RPKM).

The CAZy genes of *A. oryzae* MA1 were searched by matching the amino acid sequences of all of its CDS to those in the CAZy database in dbCAN3 using both the DIAMOND and HMMER programs (Zheng et al., 2023). Genes that were commonly identified by both programs were designated as CAZy genes of *A. oryzae* MA1. These CAZy genes were categorized into the following groups: glycoside hydrolase (GH), glycotransferase (GT), carbohydrate esterase (CE), auxiliary activity (AA), polysaccharide lyase (PL), and carbohydrate binding module (CBM). Moreover, within each CAZy category, they were subcategorized into more specific types based on their enzymatic functions. Additionally, the CAZy genes of *A. oryzae* MA1 were sorted based on their involvement in the decomposition of specific types of complex carbohydrates. The transcriptomic expressions of the CAZy categories were determined by summing the RPKM values of all genes (CDS) within each category.

2.4. Transcriptomic analysis of metabolic characteristics of *A. oryzae* MA1 during fermentation

All CDS identified in the genome of *A. oryzae* MA1 were functionally annotated based on their KEGG orthology (KO) by subjecting their amino acid sequences to BlastKOALA (<http://www.kegg.jp/blastkoala/>) analysis (Kanehisa et al., 2016) with the default parameters. The transcriptional expressions of each KEGG category during fermentation were quantified as the sum of RPKM values of all CDS assigned to each KEGG functional category.

The metabolic pathways of *A. oryzae* MA1 for complex carbohydrates, proteins, and lipids were reconstructed by integrating the

predicted KEGG pathways and the Enzyme Commission numbers of the functional genes identified in *A. oryzae* MA1. Furthermore, the presence or absence of metabolic genes in the genome was manually curated through BLASTP searches, utilizing reference protein sequences. The transcriptional expressions of genes within the reconstructed metabolic pathways during fermentation were visualized as heatmaps based on their RPKM values.

2.6. Data availability

The sequencing data of transcriptomes obtained from Illumina sequencing of doenjang-meju samples during fermentation in this study are publicly available in the NCBI Sequence Read Archive (SRA) under accession numbers SRR24566904 to SRR24566908 (NCBI BioProject accession number PRJNA970940).

2.5. Transcriptomic analysis of biosynthesis gene clusters (BGCs) of *A. oryzae* MA1 during fermentation

In *A. oryzae* MA1, BGCs responsible for secondary metabolite biosynthesis were identified using the antiSMASH database with the default parameters (Blin et al., 2023). These identified BGCs were then sorted based on their anticipated secondary metabolite types, and the transcriptional expressions for each BGC category during doenjang-meju fermentation were assessed by summing the RPKM values of core biosynthetic genes from all BGCs within each category. Moreover, the identified BGCs were cross-referenced with known secondary metabolite BGCs in the MIBiG repository (Kautsar et al., 2020). The transcriptional expressions of established secondary metabolite BGCs during doenjang-meju fermentation were evaluated using the RPKM values of their core biosynthetic genes.

3. Results

3.1. Changes in bacterial and fungal abundances during doenjang-meju fermentation

The abundance of bacteria and fungi in doenjang-meju during fermentation was determined by counting the colonies grown on TSA and PDA, respectively (Fig. 1). Initially (day 0), the bacterial count, likely attributed to *B. velezensis* MB1 inoculation, was approximately 3.7

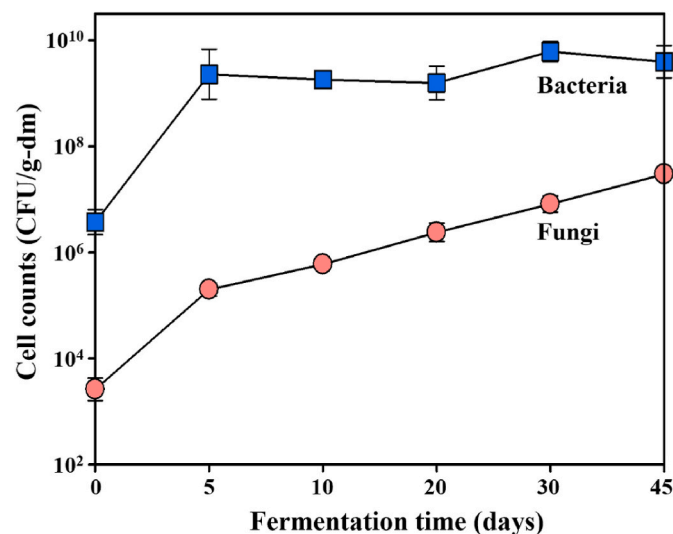


Fig. 1. Cell count profiles of bacteria and fungi in doenjang-meju inoculated with *Aspergillus oryzae* MA1 and *Bacillus velezensis* MB1 during fermentation. Data are provided as mean values $\pm$ standard errors from three measurements. CFU/g-dm, colony forming unit/gram-doenjang-meju.

$\times 10^6$ CFU/g doenjang-meju. Within the first 5 days of fermentation, the bacterial count rapidly increased to approximately 3.9×10^9 CFU/g doenjang-meju. Following this initial increase, bacterial abundance remained relatively stable until the end of fermentation. In contrast, the initial fungal count (day 0), likely attributable to *A. oryzae* MA1 inoculation, was approximately 2.6×10^3 CFU/g doenjang-meju. Fungal abundance exhibited a gradual increase throughout the fermentation period, reaching approximately 3.0×10^7 CFU/g doenjang-meju at 45 days. These bacterial and fungal profiles observed during doenjang-meju fermentation were consistent with those reported in previous studies (Han et al., 2023; Jung et al., 2014).

3.2. Transcriptomic analysis of *A. oryzae* MA1 during doenjang-meju fermentation

A total of approximately 204 million Illumina sequencing reads, ranging from approximately 36.8 to 44.1 million sequencing reads for each sample, were generated from five doenjang-meju samples collected on days 5, 10, 20, 30, and 45 (Table S1). After removing low-quality, rRNA, and tRNA sequencing reads, each sample yielded approximately 11.9–22.4 million high-quality sequencing reads. Afterward, these high-quality reads were mapped to the genome of *A. oryzae* MA1, resulting in approximately 9.3–17.3 million *A. oryzae* MA1 reads, accounting for 72.1%–90.7% of the total high-quality reads. These *A. oryzae* MA1 sequencing reads were used for the transcriptomic analysis of *A. oryzae* MA1 during doenjang-meju fermentation.

During doenjang-meju fermentation, the metabolic characteristics of *A. oryzae* MA1 were examined by mapping its sequencing reads to the CDS. This process resulted in a successful mapping of approximately 7.1–13.5 million reads, accounting for 76.1%–78.9% of the total *A. oryzae* MA1 reads (Table S1). The genome-wide transcriptional profiles, represented using heatmaps based on the RPKM values of all CDS, revealed changes in the metabolic characteristics of *A. oryzae* MA1 during doenjang-meju fermentation (Fig. S1). Notably, the hierarchical clustering of the transcriptional profiles revealed distinct alterations in the metabolic characteristics of *A. oryzae* MA1 between 10 days and 20 days.

3.3. Transcriptomic analysis of CAZy genes of *A. oryzae* MA1 during doenjang-meju fermentation

Analyzing the composition of CAZy genes within a microbe offers insights into its intrinsic capability to break down and metabolize various complex carbohydrates. Numerous studies have confirmed that *A. oryzae* is highly proficient in decomposing various types of carbohydrates (Mäkelä et al., 2018). Therefore, we conducted an analysis of the CAZy gene profile within the genome of *A. oryzae* MA1, along with an assessment of their transcriptional expressions during doenjang-meju fermentation. A total of 529 putative CAZy genes were identified in the genome of *A. oryzae* MA1, and the CAZy genes were categorized into six CAZy families: 300 genes for glycoside hydrolases (GHs), 101 genes for glycosyltransferases (GTs), 26 genes for polysaccharide lyases (PLs), 25 genes for carbohydrate esterases (CEs), 67 genes for auxiliary activities (AAs), and 10 genes for carbohydrate-binding modules (CBMs) (Fig. 2A). The substantial presence of GH genes in *A. oryzae* MA1 suggests that *A. oryzae* can proficiently hydrolyze a wide range of complex carbohydrates.

The transcriptional activities of the six CAZy families in *A. oryzae* MA1 were then analyzed during doenjang-meju fermentation (Fig. 2A). The transcriptional profiles of these CAZy families mirrored their abundance profiles in *A. oryzae* MA1 relatively well. However, the RPKM values, indicative of relative transcriptional expression, markedly decreased for CAZy genes, especially GH and AA genes, following their peak at 10 days. Conversely, the transcriptional expression of GTs exhibited an increase after 10 days. These observations suggest that carbohydrate hydrolysis is most active during the early stages of

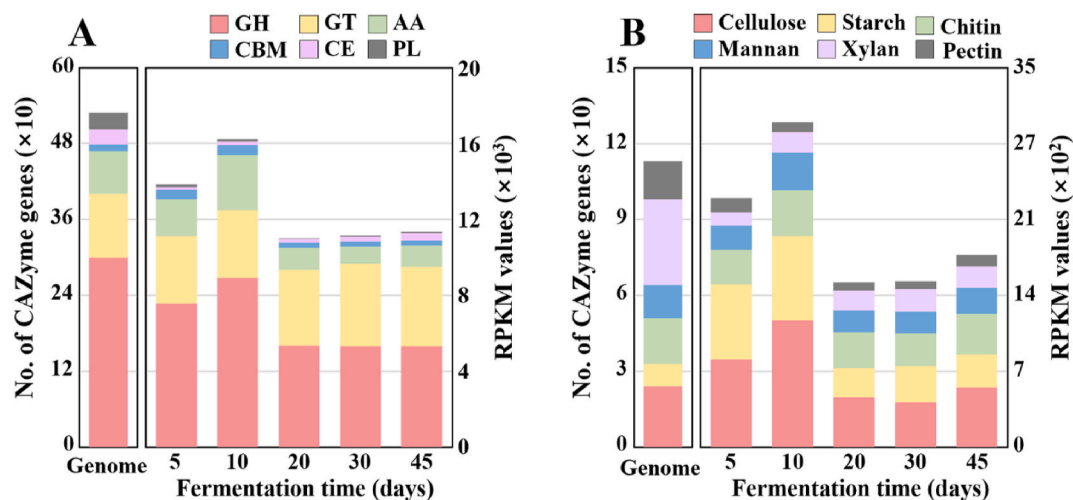


Fig. 2. Composition of carbohydrate-active enzyme (CAZy) genes sorted by (A) functional categories (GH, glycoside hydrolases; GT, glycosyltransferases; AA, auxiliary activities; CE, carbohydrate esterases; PL, polysaccharide lyases; CBM, carbohydrate-binding molecules) and by (B) the types of carbohydrates that they can break down (B), as identified in the genome of *Aspergillus oryzae* MA1, and their transcriptional expressions during doenjang-meju fermentation. The transcriptomic expressions were determined by summing the RPKM values of all genes within each category.

doenjang-meju fermentation, whereas the formation and breakdown of glycosidic linkages by GTs become more prominent as fermentation progresses. Additionally, the significant changes between days 10 and 20 were consistent with the distinct shifts in transcriptomic profiles observed in *A. oryzae* MA1 (Fig. S1).

The CAZy genes in *A. oryzae* MA1 were categorized into six functional groups based on their involvement in breaking down specific types of carbohydrates. Genes associated with xylan decomposition were the most prevalent (34 genes), followed by those involved in the decomposition of cellulose (24 genes), chitin (18 genes), pectin (15 genes), and mannan (13 genes). In contrast, the genes related to starch decomposition were the least prevalent (9 genes) (Fig. 2B). Among these, the genes associated with cellulose, starch, and chitin metabolism exhibited the highest levels of transcriptional expression. Notably, genes associated with starch decomposition showed high expression levels despite their low abundance in *A. oryzae* MA1. However, their expression significantly decreased after 10 days. These observations suggest that cellulose and starch decomposition may be particularly active processes during the early fermentation of doenjang-meju. However, genes associated with the decomposition of xylan and pectin showed consistently low expression levels throughout the entire fermentation period, indicating that xylan and pectin decomposition is less active during doenjang-meju fermentation.

To further characterize the ability of *A. oryzae* to decompose various complex carbohydrates during doenjang-meju fermentation, we performed a comprehensive functional analysis of the relevant CAZy genes, in addition to examining their respective transcriptional expressions (Fig. 3). Among the CAZy genes, GH17 (glucan β -1,3-glucosidase), GH72 (β -1,3-glucanotransglycosylase), GH16 (chitin β -1,6-glucanotransglycosylase), and GH13 (α -amylase) in the GH category; GT20 (α , α -trehalose-phosphate synthase), GT2 (chitin synthase), GT4 (trehalose phosphorylase), and GT15 (glycolipid 2- α -mannosyltransferase) in the GT category; CE9 (*N*-acetylglucosamine 6-phosphate deacetylase), CE12 (acetyltransferase), CE5 (cutinase), CE4 (chitin deacetylase), and CE8 (pectin methyltransferase) in the CE category; AA3 (alcohol oxidase), AA9 (lytic cellulose monooxygenase), AA2 (cytochrome-c peroxidase), and AA11 (lytic chitin monooxygenase) in the AA category; PL1 (pectin lyase and pectate lyase), PL3 (pectate lyase), PL26 (rhamnogalacturonan exolyase), and PL4 (rhamnogalacturonan endolyase) in the PL category; and CBM21 (starch-binding domain) and CBM87 (galactosaminogalactan-binding domain) in the CBM category were highly expressed in *A. oryzae* MA1 during doenjang-meju fermentation (Fig. 3).

However, their transcriptional profiles varied depending on the fermentation stage. For example, the GH17, GH16, GH13, GH5, CE12, AA3, AA9, PL1, and CBM21 genes were highly expressed during the early fermentation stages (5 and 10 days), with their expressions decreasing as fermentation progressed. Conversely, the transcriptional expressions of the GT20, GT4, CE8, and CE9 genes remained low during the early fermentation stages but increased as the fermentation progressed. Additionally, the transcriptional expressions of the GH72, GT2, GH15, and PL4 genes remained relatively constant throughout the entire fermentation process. These results suggest that the type of carbohydrates preferentially decomposed by *A. oryzae* in doenjang-meju fermentation varies depending on the fermentation stage, as illustrated in Fig. 2B.

Trehalose is known to be an essential compatible solute that enables *Aspergillus* to cope with drought stress (Al-Bader et al., 2010). The transcriptional expression of GT20 (α , α -trehalose-phosphate synthase) genes, which are involved in trehalose biosynthesis, increased as fermentation progressed (Fig. 3B). This suggests that the heightened expression of GT20 towards the end of fermentation process may be related to trehalose accumulation in *A. oryzae* to counteract drought stress, as water content in doenjang-meju gradually decreases during fermentation (Han et al., 2023; Jung et al., 2014). Additionally, the transcriptional gene expressions of CE9 (*N*-acetylglucosamine 6-phosphate deacetylase), involved in the assimilation and recycling of *N*-acetylglucosamine, a major cell wall component in bacterial cells, rapidly increased as fermentation progressed (Fig. 3C). This might be linked to bacterial growth during doenjang-meju fermentation. The transcriptional expression of AA3 (alcohol oxidase) genes, involved in primary alcohol oxidation, exhibited an increase at 10 days but decreased thereafter as fermentation progressed. This trend is likely linked to the production of primary alcohols from sugars by anaerobic fermentation processes driven due to the high-water content of doenjang-meju during the initial fermentation stages. Lastly, the transcriptional expression of CBM21 genes, involved in starch-binding, remained high during the early fermentation period and decreased as fermentation progressed (Fig. 3F), likely indicating the gradual depletion of starch during the fermentation process.

Taken together, our results indicate that the broad classification and expression analysis of CAZy genes, as illustrated in Fig. 2, may not comprehensively capture the carbohydrate decomposition characteristics of *A. oryzae*. Instead, a more detailed analysis of CAZy genes, as depicted in Fig. 3, offers clearer insights into the complex carbohydrate

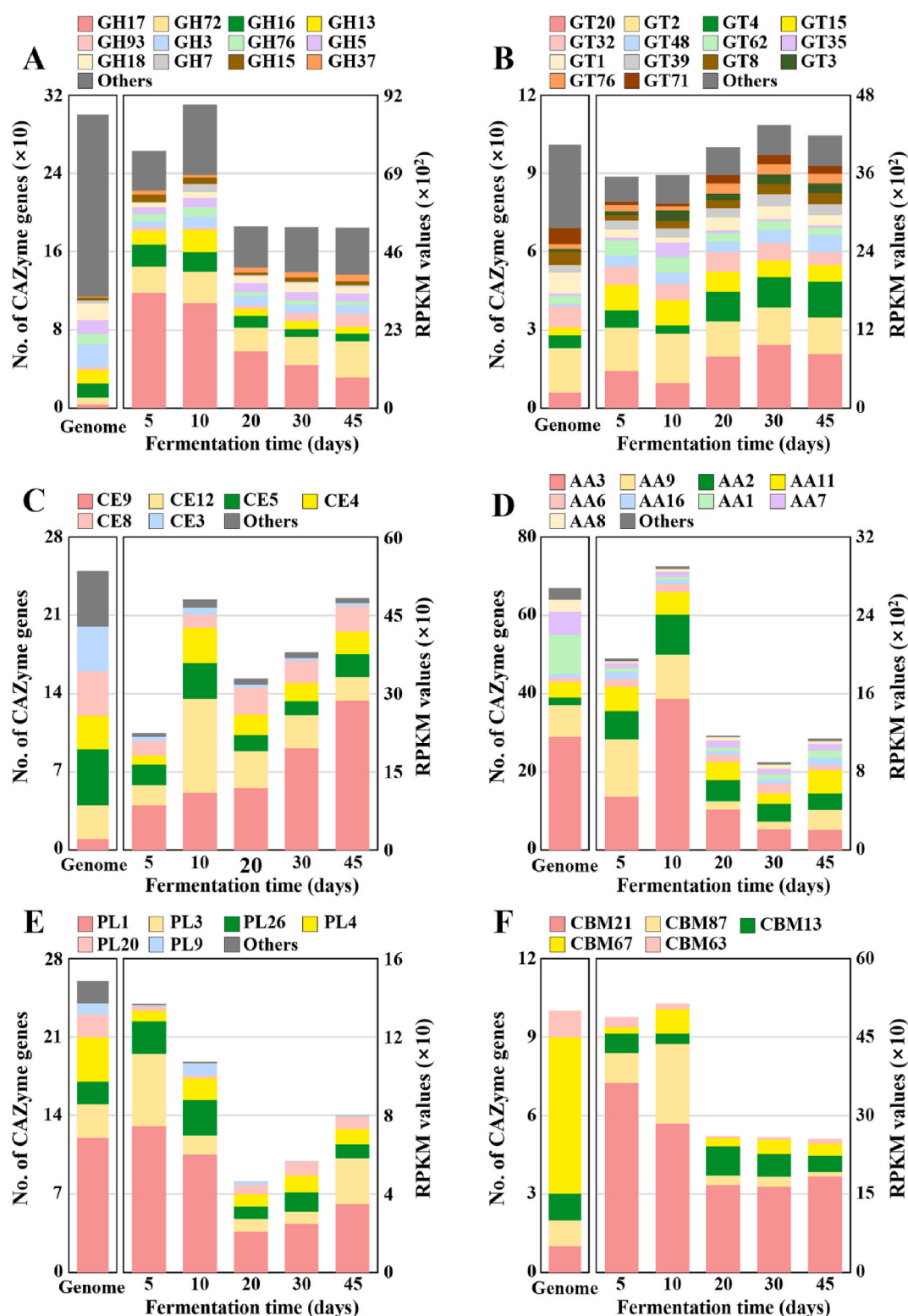


Fig. 3. Functional classification of carbohydrate-active enzyme (CAZy) genes belonging to glycoside hydrolase (GH; A), glycosyltransferase (GT; B), carbohydrate esterase (CE; C), auxiliary activity (AA; D), polysaccharide lyase (PL; E), and carbohydrate-binding module (CBM; F) in *Aspergillus oryzae* MA1 and their transcriptional expressions during doenjang-meju fermentation. The transcriptomic expressions were determined by summing all RPKM values of functionally identical genes. The subcategory “Others” encompasses functional subcategories with relative RPKM values below 3.0% across all samples.

decomposition patterns of *A. oryzae* during doenjang-meju fermentation.

3.4. Transcriptomic analysis of metabolic characteristics of *A. oryzae* MA1 during doenjang-meju fermentation

The identified CDS in *A. oryzae* MA1 were sorted into functional KEGG categories, and their transcriptional expressions were examined

during doenjang-meju fermentation. The KEGG distributions of the mRNA sequencing reads of *A. oryzae* MA1 differed from those of the CDS from *A. oryzae* MA1 (Fig. 4). Notably, based on the relative abundances of CDS in the KEGG categories, the translation and energy metabolism categories at the secondary level, which may be related to cell growth, exhibited high expression levels, particularly during the initial fermentation period (Fig. 4A), indicating active cell growth of *A. oryzae* MA1. The transcriptional expressions decreased slightly after 10 days, indicating a minor slowdown in growth. However, the transcriptional

expressions of KEGG metabolic categories, including carbohydrate metabolism, lipid metabolism, and nucleotide metabolism, at the secondary level were relatively constant, suggesting that the metabolic features remained relatively constant throughout the entire fermentation period. These results align with the fungal growth curve presented in Fig. 1, which showed an initial slightly rapid growth, followed by nearly constant growth.

The transcriptional expressions of KEGG subcategories for carbohydrates at the tertiary level during doenjang-meju fermentation were also

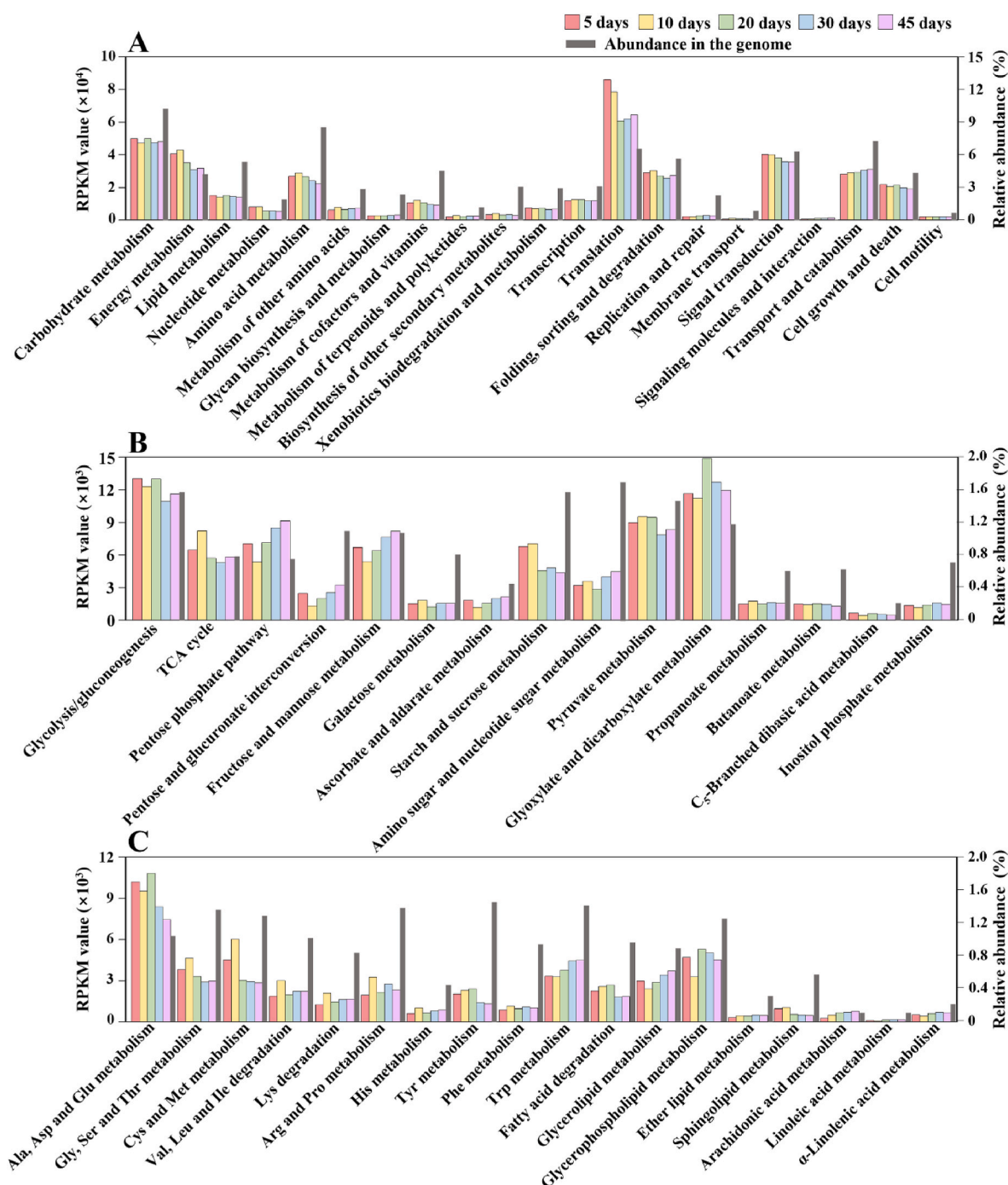


Fig. 4. Transcriptional expression of KEGG functional genes in *Aspergillus oryzae* MA1 during doenjang-meju fermentation. All coding sequences were categorized at the secondary level (A), and carbohydrate metabolic (B) and amino acid and lipid metabolic (C) genes were further categorized at the tertiary level (C), and their transcriptional expressions were analyzed throughout the fermentation process. Transcriptomic expressions were quantified by summing the RPKM values of all genes within each KEGG category. The gray bars on the right side of each category depict the relative abundance (%) of functional genes (CDS) from *A. oryzae* MA1 within each KEGG category.

investigated (Fig. 4B). The genes within certain subcategories such as glycolysis/gluconeogenesis, the pentose phosphate pathway, fructose and mannose metabolism, and glyoxylate and dicarboxylate metabolism exhibited high expressions compared to those under other subcategories. In contrast, the genes in the galactose metabolism, starch and sucrose metabolism, pentose and glucuronate interconversion, amino sugar and nucleotide sugar metabolism, propanoate metabolism, butanoate metabolism, and inositol phosphate metabolism subcategories exhibited low expressions. These results suggest that *A. oryzae* MA1 may actively metabolize glucose and fructose/mannose as major carbon sources during doenjang-meju fermentation, whereas the metabolism of galactose, starch/sucrose, and pentose/glucuronate may be relatively

less pronounced. While the transcriptional expression of carbohydrate metabolism at the secondary level remained relatively stable throughout the entire fermentation period, at the tertiary level, the transcriptional profiles exhibited variations depending on the specific subcategories. Specifically, the transcriptional expression of glycolysis/gluconeogenesis and starch and sucrose metabolism subcategories decreased as fermentation progressed. In contrast, the transcriptional expression of fructose and mannose metabolism, pentose and glucuronate interconversion, and pentose phosphate pathway subcategories increased as fermentation progressed. The transcriptional analysis of KEGG subcategories for amino acids

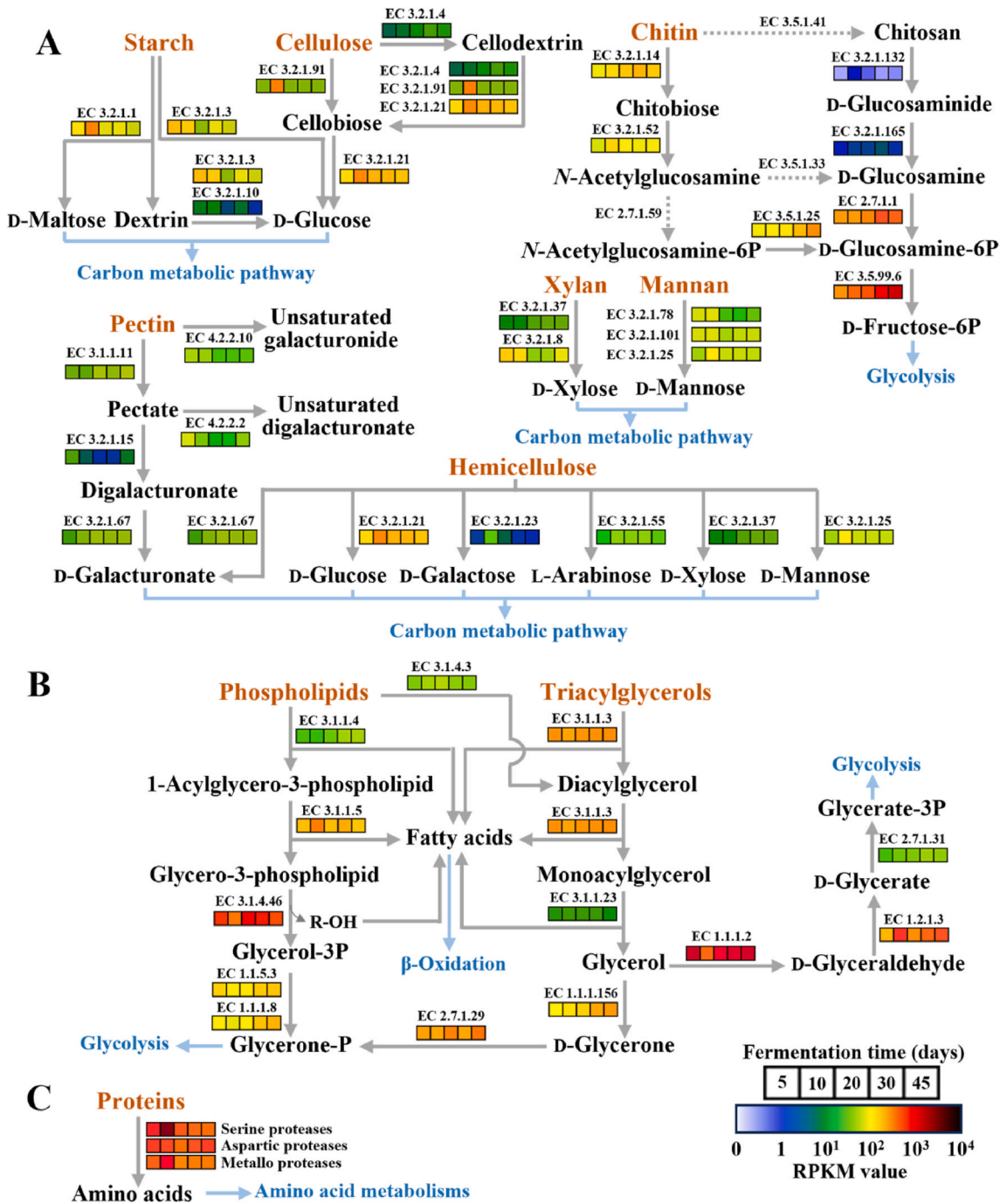


Fig. 5. Proposed hydrolysis pathways of complex carbohydrates (A), lipids (B), and proteins (C) in *Aspergillus oryzae* MA1 and transcriptional expressions of corresponding genes during doenjang-meju fermentation. The transcriptional expression levels of metabolic genes during fermentation were visualized using heatmaps based on their RPKM values. The dotted arrows represent metabolic pathways that are not identified in *A. oryzae* MA1.

and lipids at the tertiary level revealed that the subcategory of alanine, aspartate, and glutamate metabolism exhibited elevated expression levels compared to other subcategories (Fig. 4C). This result may be linked to the high concentrations of alanine, glutamate, and aspartate observed in doenjang-meju (Kim et al., 2022). Furthermore, with the exception of certain subcategories such as tryptophan metabolism, glycerolipid metabolism, and arachidonic acid metabolism, the transcriptional expression of most subcategories decreased as fermentation progressed.

3.5. Reconstruction of metabolic pathways of *A. oryzae* MA1 and transcriptomic analysis during doenjang-meju fermentation

To investigate the metabolic characteristics of *A. oryzae* MA1 during doenjang-meju fermentation, we reconstructed the metabolic pathways of *A. oryzae* MA1 for carbohydrates, lipids, and proteins, which are key macromolecular components of soybeans. Afterward, we conducted a transcriptomic analysis for these pathways during doenjang-meju fermentation (Fig. 5). The transcriptomic analysis of carbohydrate metabolic pathways revealed active expression in the decomposition pathways for starch, cellulose, hemicellulose, pectin, xylan, mannan, and chitin (Fig. 5A), which was consistent with the findings in the CAZY analysis (Fig. 3). Genes responsible for the decomposition of starch, cellulose, hemicellulose, xylan, and mannan exhibited high expression levels during the early stages of fermentation, especially at 10 days. Specifically, genes involved in the hydrolysis of starch, cellulose, and hemicellulose into glucose exhibited high transcriptional expression levels. However, despite being one of the major components of soybeans, pectin metabolic genes exhibited generally low expression levels throughout the entire fermentation process, with a slight increase in the later stages.

The transcriptional expressions of genes involved in the hydrolysis of hemicellulose into D-glucose, D-galactose, L-arabinose, D-xylose, D-mannose, and D-galacturonate exhibited notable disparities depending on the specific genes involved. Specifically, the gene encoding β -galactosidase (EC 3.2.1.23), which is involved in galactose production, displayed very low transcriptional expression except on day 10. Additionally, the transcriptional expressions of genes encoding α -L-arabinofuranosidase (EC 3.2.1.55) and exo-xylosidase (EC 3.2.1.37), involved in the production of L-arabinose and D-xylose from hemicellulose, were high during the late fermentation stages, whereas other genes exhibited high transcriptional expressions during the early stages of fermentation.

Genes associated with chitin decomposition exhibited relatively high levels of transcriptional expression. However, the gene responsible for producing chitosan from chitin was not identified in *A. oryzae* MA1, which is potentially a major factor contributing to the very low transcriptional expression of genes involved in the subsequent production of D-glucosaminide and D-glucosamine from chitosan. In contrast, although the genes responsible for converting N-acetylglucosamine to N-acetylglucosamine-6P (EC 3.5.1.25) or D-glucosamine (EC 3.5.1.33) were not identified in *A. oryzae* MA1, genes involved in the conversion of N-acetylglucosamine-6P to D-glucosamine-6P, and subsequently to D-fructose-6P, exhibited notably high levels of transcriptional expression. These results suggest the potential involvement of other microbes, such as *Bacillus* (Gaugué et al., 2013), in the conversion of N-acetylglucosamine to N-acetylglucosamine-6P or D-glucosamine during doenjang-meju fermentation.

The transcriptomic analysis of lipid metabolic pathways identified triacylglycerols and phospholipids as the primary lipid compounds actively metabolized during doenjang-meju fermentation (Fig. 5B). However, unlike the transcriptomic expression of carbohydrate metabolic pathways, the transcriptomic expressions of lipid metabolic pathways generally increased as the fermentation progressed. These findings suggest that lipids become a vital resource in the later stages of fermentation when carbohydrates become depleted in doenjang-meju

fermentation. The transcriptional expression of lipase (EC 3.1.1.3) was notably higher than that of phospholipase (EC 3.1.1.4), likely due to the presence of free triglycerides in soybeans, whereas phospholipids are integrated within cell membrane components. In fact, our findings demonstrated that the transcriptional expression of genes involved in the metabolic pathway subsequent to phospholipid hydrolysis by phospholipase was markedly elevated. In *A. oryzae* MA1, three kinds of protease genes responsible for the hydrolysis of proteins, which are prominent constituents of soybeans, were identified, and these genes exhibited consistently high levels of transcriptional expression throughout the entire fermentation process (Fig. 5C). This observation suggests that *A. oryzae* actively engages in the hydrolysis of proteins during doenjang-meju fermentation.

For a more detailed analysis of the carbohydrate metabolisms of *A. oryzae* in doenjang-meju, the carbohydrate metabolic pathways of *A. oryzae* MA1 were reconstructed, and their transcriptional expressions were examined throughout the fermentation process (Fig. 6). The reconstructed metabolic pathways revealed that *A. oryzae* MA1 employs complete glycolysis, 6-phosphogluconate/phosphoketolase, and pentose phosphate pathways, pyruvate metabolism, and the tricarboxylic acid (TCA) cycle capable of metabolizing diverse carbon compounds, highlighting its versatile metabolic capacity and inherent aerobic characteristics. The transcriptome analysis revealed that genes associated with the metabolism of 6-carbon monosaccharides, namely D-glucose, D-fructose, D-mannose, and L-sorbose (except for D-galactose), along with glycolysis and the TCA cycle, exhibited high expression levels throughout the entire fermentation process (Fig. 6). Additionally, these transcriptomic expression levels generally increased as the fermentation progressed. However, genes linked to the metabolism of disaccharides, such as sucrose and D-maltose, showed relatively low transcriptomic expression levels. Similarly, genes involved in the metabolism of pentoses, including L-arabinose, D-ribose, and D-xylose (with slightly higher expression of metabolic genes for D-xylose), displayed relatively low expression levels compared to 6-carbon monosaccharides. Nevertheless, these levels increased as the fermentation progressed, following a pattern similar to that observed with 6-carbon monosaccharides. These results suggest that *A. oryzae* tends to preferentially metabolize 6-carbon monosaccharides over other sugar compounds. On the other hand, intriguingly, our transcriptome analyses revealed that *A. oryzae* MA1 also possesses metabolic routes related to lactate, acetate, and ethanol, all of which are representative fermentation products, and these genes also exhibited high transcriptional expressions over the entire fermentation period.

3.6. Transcriptomic analysis of BGCs of *A. oryzae* MA1 during doenjang-meju fermentation

A. oryzae is well-known for its capacity to produce a wide range of secondary metabolites (Uka et al., 2020). Therefore, the BGCs in the genome of *A. oryzae* MA1 were scrutinized, and their transcriptional expressions were examined throughout the doenjang-meju fermentation process (Fig. 7). A total of 52 BGCs were identified, spanning various structural categories: polyketide synthase (PKS, 14), non-ribosomal peptide synthetase (NRPS, 10), NRPS-like (10), ribosomally synthesized and post-translationally modified peptide-like (RiPP-like, 5), indole (3), terpene (6), hybrid 1 (harboring a chimeric core-biosynthetic gene of PKS and NRPS, 2), hybrid 2 (harboring two core-biosynthetic genes of terpene and NRPS, 1), and betalactone (1) (Fig. 7A). Among these, 25 BGCs were associated with known secondary metabolites listed in the MIBiG database (Table S2). The conspicuous abundance of BGCs in *A. oryzae* MA1 underscores its inherent capability to produce diverse secondary metabolites.

The transcriptional expressions revealed that the total RPKM values of the BGCs in *A. oryzae* MA1 remained relatively constant throughout the entire fermentation period, with day 10 being the only exception (Fig. 7A). Interestingly, the total RPKM values were lowest on day 10 of

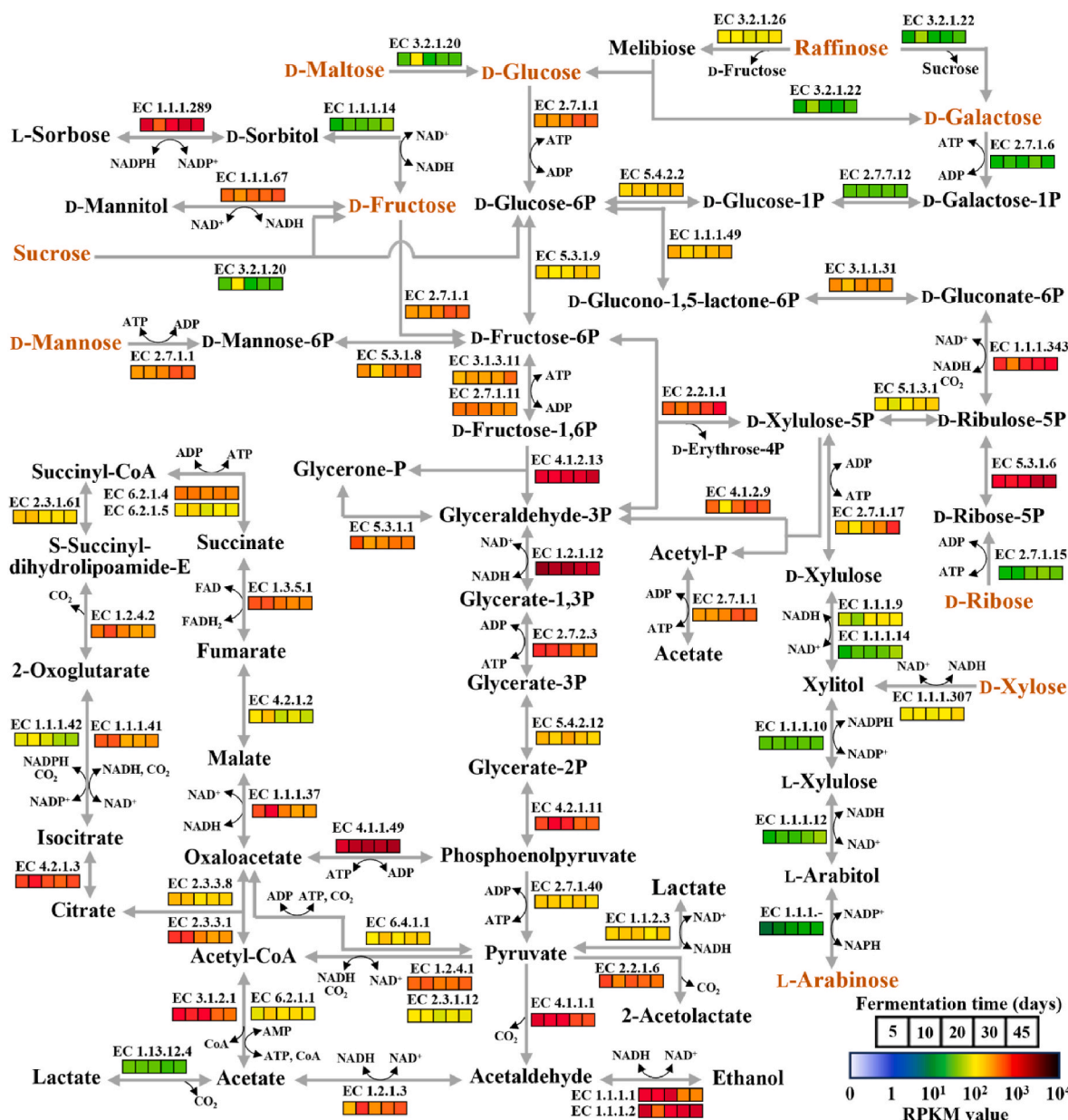


Fig. 6. Proposed metabolic pathways of *Aspergillus oryzae* MA1 for carbon compounds and the transcriptional expressions of corresponding metabolic genes during doenjang-meju fermentation. The transcriptional expression levels of metabolic genes during fermentation were visualized using heatmaps based on their RPKM values.

fermentation, coinciding with the highest expression levels of CAZy genes, which may be related to the growth of *A. oryzae* MA1, as depicted in Fig. 1. This observation suggests that the production of secondary metabolites may be reduced during periods of active CAZy production, likely indicative of rapid growth. The transcriptional levels of BGC categories during doenjang-meju fermentation displayed varying degrees of correlation with their abundance in *A. oryzae* MA1. BGCs classified under the PKS, NRPS-like, NRPS, and terpene categories, which are abundant in *A. oryzae* MA1, exhibited high transcriptional expression levels during doenjang-meju fermentation. In contrast, BGCs categorized under RiPP-like and indole showed considerably lower transcriptional levels compared to their abundance, and the betalactone category, which consisted of only one BGC, exhibited high transcriptional expression.

The transcriptional expressions of 25 BGCs associated with known secondary metabolites were individually assessed during doenjang-meju fermentation, using the expression levels of their core biosynthesis genes

(Fig. 7B–Table S2). The transcriptional expression levels of these BGCs varied significantly depending on the BGCs during doenjang-meju fermentation. Notably, YWA1 synthesis-related BGC (assessed through the expression of the *alb1* gene), which is involved in the synthesis of precursors of fungal conidial pigment, exhibited the highest transcriptional expression during doenjang-meju fermentation. The expression of this gene was followed by those of squalstatin S1 (*phps1*), penicillin (*pcbAB*), choline (*atr*), 6-methylsalicylic acid (*atX*), clavarinic acid (*occ*), 2,4'-dihydroxy-3'-methoxypropionophenone (*ppsB*), actinopolymorphol C (*lnaA*), and metachelin C (*mrsidD*)-related BGCs. Other BGCs displayed low transcriptional expression, with RPKM values of 10 or less.

The transcriptional expression profiles of BGCs during doenjang-meju fermentation exhibited substantial variations, dependent on the specific BGCs. The transcriptional expressions of core-biosynthetic genes in BGCs related to the synthesis of squalstatin S1 and 2,4'-dihydroxy-3'-methoxypropionophenone gradually declined as the fermentation progressed. Conversely, the transcriptional expressions of BGCs related to

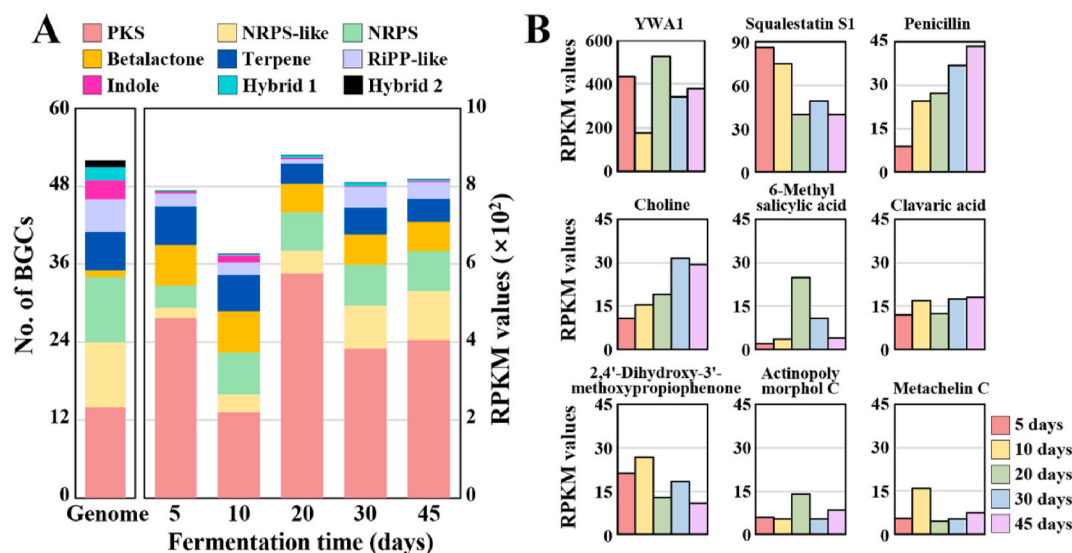


Fig. 7. Transcriptional expressions of biosynthesis gene clusters (BGCs) in *Aspergillus oryzae* MA1 during doenjang-meju fermentation. BGCs in panel A were categorized based on the types of secondary metabolites, and their transcriptional expressions were determined by summing the RPKM values of core biosynthetic genes in all BGCs within each category. The transcriptional expressions of BGCs in panel B were assessed by the RPKM values of their core biosynthetic genes indicated in Table S1. Core-biosynthetic genes with RPKM values consistently below 10 throughout the fermentation period were not indicated. Abbreviations: PKS, polyketide synthase; NRPS, non-ribosomal peptide synthetase; RiPP, ribosomally synthesized and post-translationally modified peptides.

the synthesis of penicillin and choline exhibited a gradual increase as fermentation advanced. On the other hand, the transcriptional expressions of BGCs related to the synthesis of YWA1, 6-methylsalicylic acid, actinopolymorphol C, and metachelin C were highest during the middle of fermentation (10 or 20 days). Remarkably, the BGC associated with YWA1 synthesis, which exhibited the highest transcriptional expression among the BGCs, exhibited an abrupt decrease on day 10 of fermentation, unlike the other BGCs. Given that the total RPKM value for BGCs was at its lowest on day 10 (Fig. 7A), our findings suggest that this dip on day 10 of fermentation was primarily attributed to the YWA1 synthesis-related BGC. This observation suggests that the production of the YWA1-related secondary metabolite might be considerably reduced during periods of active CAZy production in *A. oryzae* MA1 (Fig. 2), whereas the production of secondary metabolites by other BGCs might not be affected.

4. Discussion

The filamentous fungus *A. oryzae* is widely recognized for its pivotal role in the preparation of essential starting ingredients such as koji, nuruk, and doenjang-meju, which is crucial for the production of diverse fermented foods, including soy sauces, fermented soybean pastes, rice wine, and baiju (Han et al., 2024; Kim et al., 2022; Liu et al., 2022; Park et al., 2019; Yang et al., 2013; Yasui et al., 2020). Acknowledged as a primary fermentation catalyst, *A. oryzae* efficiently degrades complex molecules, including carbohydrates, proteins, and lipids, into simpler compounds such as sugars, amino acids, and free fatty acids during fermentation. This proficiency is attributed to its high production of various hydrolytic enzymes (Hong et al., 2015; Zhao et al., 2019). Subsequently, these simplified compounds undergo further fermentation by other microbes, including yeasts and lactic acid bacteria. While prior studies have mainly focused on the hydrolytic enzymatic activities of *A. oryzae* strains during fermentation (Bal et al., 2016; Li, Sun, et al., 2023), their metabolic features during fermentation have not been comprehensively explored. To bridge this knowledge gap, we conducted a genome-wide transcriptomic analysis to systematically investigate the metabolic features of *A. oryzae* during doenjang-meju fermentation. Specifically, to thoroughly scrutinize the fermentative metabolic features of *A. oryzae* in doenjang-meju, we conducted a comprehensive

reconstruction and transcriptomic analysis of detailed metabolic pathways for complex carbohydrates, lipids, and proteins, along with the transcriptomic analyses of CAZy and BGC genes, in *A. oryzae* throughout the entirety of the fermentation period.

Although the characteristics of isolated single microbes may differ from those observed in coexistence with other microbes, previous studies on the fermentative metabolic features of microbes through transcriptome analysis have predominantly concentrated on individual isolated microbes (Canon et al., 2020; Zhang et al., 2022; Zhong et al., 2018). Notably, the fermentation of traditional foods such as koji, nuruk, and doenjang-meju often involves the cooperative interaction of bacteria and fungi with distinct metabolic characteristics (Chun et al., 2021). These observations highlight the importance of studying these microbes within the context of the microbial consortia present in those foods.

The quantification of viable bacterial and fungal cells during the doenjang-meju fermentation revealed a substantial predominance of bacterial abundances, which was likely predominantly attributable to *B. velezensis*, surpassing fungal abundances, primarily attributed to *A. oryzae*, by approximately more than 100 times (Fig. 1). However, despite the lower cell counts, fungal transcriptomes constituted a significant proportion, ranging from 72.1% to 90.7% of the total transcriptomes (Table S1). This observation may be attributed to the possibility that fungal genes exhibit higher expression levels compared to bacterial genes or that fungal mRNA transcripts possess considerably longer intracellular half-lives than bacterial mRNA transcripts. These findings suggest that assessing the contribution of bacteria and fungi to fermentation based on viable cell numbers can introduce substantial biases.

The transcriptional profiles of *A. oryzae* revealed distinct alterations in its metabolic features between 10 and 20 days of doenjang-meju fermentation (Fig. S1). Particularly, we observed a significant decrease in the transcriptional levels of CAZy genes in *A. oryzae*, which coincided with a significant change in the transcriptional profiles of CAZy genes between days 10 and 20 (Figs. 2 and 3). Although the transcriptional expression of CAZy genes (Fig. 2), which provide carbon sources necessary for the growth of *A. oryzae*, decreased after 20 days, *A. oryzae* continued to grow after 20 days (Fig. 1). It is presumed that the high activity of CAZy genes during the early fermentation stages of doenjang-meju resulted in a substantial production and accumulation of free

sugars and *A. oryzae* sustained its growth after 20 days by utilizing these accumulated free sugars (Kim et al., 2002). However, when evaluating the metabolic features of *A. oryzae* during fermentation, it is essential to consider its abundance in doenjang-meju. Despite the decline in the relative transcriptional expressions of CAZy genes after 10 days, the increased abundance of *A. oryzae* at the end of doenjang-meju fermentation (Fig. 1) suggests that the overall CAZy activities of *A. oryzae* may be greater at the end of fermentation.

Soybeans are composed of approximately 30%–35% carbohydrates, 40% proteins, and 20% lipids, and among them, carbohydrates consist of starch, cellulose, hemicellulose (containing mannan and xylan), and pectin as the main polysaccharides (Medic et al., 2014). Throughout the process of doenjang-meju fermentation, microbes, including fungi and bacteria, are known to hydrolyze these soybean components, thereby enhancing the nutritional and taste values of fermented foods. Notably, fungi, including *A. oryzae*, are recognized to be closely related to the breakdown of carbohydrates during fermentation (Gil et al., 2022; Hong et al., 2015). Our CAZy gene analysis also revealed that *A. oryzae* possesses a multitude of CAZy genes capable of decomposing diverse carbohydrates, such as cellulose, starch, chitin, hemicellulose (mannan, xylan), and pectin (Fig. 2). These findings highlight the proficiency of *A. oryzae* for the decomposition of soybean carbohydrates.

In the context of doenjang-meju fermentation, our transcriptomic analysis demonstrated that *A. oryzae* may actively decompose cellulose and starch in soybeans, generating glucose (Fig. 2B and 5A). Furthermore, the β -glucosidase gene (EC 3.2.1.21) in *A. oryzae* responsible for the hydrolysis of hemicellulose to produce glucose also exhibited high transcriptional expression during doenjang-meju fermentation (Fig. 5A). The glycolysis pathway in *A. oryzae* responsible for metabolizing glucose, the primary decomposition product during the fermentation process, also exhibited increased transcriptional expression (Fig. 6). These findings suggest that glucose serves as the predominant carbon compound metabolized by *A. oryzae* during doenjang-meju fermentation.

The expression of many CAZy genes, including GH13 (α -amylase), GH5 (cellulase), AA9 (lytic cellulose monooxygenase), PL1 (pectin lyase and pectate lyase), and PL3 (pectate lyase) genes, may be closely related to the decomposition of complex carbohydrates of soybeans by *A. oryzae* MA1 during doenjang-meju fermentation. However, not all of the examined CAZy genes are involved in the decomposition of complex carbohydrates of soybeans. For instance, the expression of GH17 (glucan β -1,3-glucosidase) and GH72 (β -1,3-glucanotransglycosylase) genes, which are involved in modifying and hydrolyzing β -1,3-glucan essential for softening the cell wall for germination and branching in filamentous fungi (Mouyna et al., 2013), may be primarily associated with the cell growth of *A. oryzae* rather than carbohydrate decomposition. Furthermore, despite the absence of chitin in soybeans, genes associated with chitin metabolism exhibited relatively high levels of transcriptional expression. This occurrence is likely attributed to the composition of the fungal cell wall, which is primarily chitin-based, and not influenced by soybean components (de Nobel et al., 2000). Notably, the high transcriptional expression of GH16 (chitin β -1,6-glucanotransglycosylase), associated with fungal cell wall remodeling (Arroyo et al., 2016), and GT2 (chitin synthase) genes may also be linked to the rapid cell growth of *A. oryzae*, rather than active chitin decomposition, particularly during the early fermentation period (Fig. 3A and B).

Intriguingly, metabolic pathway analysis revealed that *A. oryzae* possesses metabolic genes associated with L-sorbose, D-mannitol, and D-sorbitol. Notably, these genes exhibited elevated transcriptional expressions throughout the entire fermentation process, even though L-sorbose, D-mannitol, and D-sorbitol are not constituents of soybeans, and they are not produced through the carbohydrate hydrolysis process in doenjang-meju fermentation (Figs. 5 and 6). These findings suggest that the transcriptional expressions of these genes may be directed towards production rather than the metabolization of these compounds, suggesting that they are potentially produced during doenjang-meju

fermentation. Additionally, the metabolic pathway analysis indicated that *A. oryzae* possesses metabolic routes related to lactate, acetate, and ethanol, all of which are representative fermentation products, and these genes consistently displayed high transcriptional expressions throughout the entire fermentation period. However, given that *A. oryzae* is strictly aerobic and lacks fermentation capabilities, the transcriptional expressions of these genes may be directed towards the metabolic processes of lactate, acetate, and ethanol (Han et al., 2023), which are typically produced by other fermentative bacteria, rather than their own production.

The prolific production of secondary metabolites by *Aspergillus* strains, capable of influencing the growth of other microorganisms and human health, presents intriguing possibilities for *A. oryzae* in various facets of doenjang-meju fermentation (Han et al., 2024; Keller, 2019). Our analysis of BGCs and their transcriptional expression in *A. oryzae* MA1 revealed potential variations in the production of diverse secondary metabolites during doenjang-meju fermentation (Fig. 7). While our findings suggest *A. oryzae*'s capability for secondary metabolite production, the actual quantities and their impacts on doenjang-meju microbial communities and human health remain unexplored. This knowledge gap emphasizes the need for further studies to quantify secondary metabolite production during doenjang-meju fermentation and understand its potential effects on microbial communities and human health. Addressing these aspects would significantly contribute to a more comprehensive understanding of the intricate dynamics of microbial communities in *A. oryzae*-mediated fermentations, which has implications for both food fermentation processing and human health.

In summary, this study comprehensively investigated the overall metabolic features of *A. oryzae* through transcriptomic analysis spanning the entire doenjang-meju fermentation period. Notably, the transcriptomic analysis of the CAZy genes of *A. oryzae* provided insights into its metabolic characteristics for various macromolecules in soybeans, especially complex carbohydrates, during doenjang-meju fermentation. Additionally, our study suggests that *A. oryzae* can produce diverse secondary metabolites throughout the fermentation process, thereby influencing both the microbial communities of doenjang-meju and human health. This study provides comprehensive insights into the metabolic traits of *A. oryzae*, advancing our understanding not only of doenjang-meju fermentations but also various fermentation processes, including koji and nuruk fermentations, in which *A. oryzae* plays a crucial role.

CRediT authorship contribution statement

Dong Min Han: Writing – original draft, Methodology, Investigation. **Ju Hye Baek:** Methodology, Investigation. **Dae Gyu Choi:** Software, Investigation. **Che Ok Jeon:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors have no conflicts of interest.

Data availability

No data was used for the research described in the article.

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Appendix B. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.lwt.2024.116740>.

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