

Article

Genome-Wide Analysis of the *YUCCA* Gene Family in Wheat and the Potential Roles of *TaYUCCA19* and Its Homologs in Male Reproductive Development

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Abstract

YUCCA belongs to the flavin-containing monooxygenases and catalyzes the rate-limiting step in endogenous auxin biosynthesis, thereby regulating local auxin homeostasis and participating in diverse aspects of plant growth, development, and physiological processes. However, the relationship between the *YUCCA* genes and male fertility regulation in wheat remains unclear. In this study, we identified 64 *TaYUCCA* genes through whole-genome analysis and classified them into three clades, each of which is conserved in motif composition and gene structure. A synteny analysis indicated that family expansion was primarily driven by segmental duplication and tandem duplication, and Ka/Ks analysis suggested that all members are under purifying selection. An analysis of the expression patterns showed that the *TaYUCCA* genes displayed differential expression across various tissues and reproductive developmental stages. In the temperature-sensitive male-sterile wheat line YS3038, *TaYUCCA19*, *TaYUCCA22*, and *TaYUCCA25* were specifically highly expressed at the uninucleate pollen stage under fertile conditions. The silencing of *TaYUCCA19* resulted in abnormal pollen morphology and a significant reduction in the seed set rate, indicating that it is a key gene required for normal pollen development in wheat. Overall, this study systematically characterizes the wheat *YUCCA* gene family and provides the first functional evidence of *TaYUCCA* genes in male reproductive development, offering an important foundation for studies on wheat male sterility mechanisms and the exploitation of heterosis.

Keywords: wheat; *YUCCA* gene family; male sterility



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1. Introduction

Wheat (*Triticum aestivum* L.) is one of the most important staple crops worldwide [1], and hybrid breeding is considered a key approach to increase the yield per unit area and improve agronomic traits in wheat [2]. However, as a self-pollinating crop, the effective utilization of heterosis in wheat still faces many challenges [3,4]. One of the critical prerequisites for successful wheat hybrid breeding is the establishment of a stable and efficient male-sterile system, which aims to prevent self-fertilization while allowing the female gametes to be pollinated by selected paternal lines [5]. Therefore, elucidating the molecular mechanisms underlying male sterility is critical for promoting the efficient utilization of heterosis in wheat.

Male-sterile phenotypes often result from abnormalities in anther or pollen development, and previous studies have shown that auxin plays a central regulatory role in

stamen/anther development [6,7]. Indole-3-acetic acid (IAA) is the predominant form of auxin in plants, and its biosynthesis primarily relies on the tryptophan (Trp)-dependent pathway, among which the indole-3-pyruvic acid (IPA) pathway is considered the main and physiologically most relevant route in plants [8,9]. In this pathway, Trp is first converted into IPA via a reversible transamination reaction catalyzed by tryptophan aminotransferases [10], and IPA is subsequently converted into IAA through an irreversible oxidative decarboxylation reaction catalyzed by the *YUCCA* family of flavin-containing monooxygenases [11–13]. Previous studies have demonstrated that this reaction constitutes a key regulatory step of auxin biosynthesis across multiple tissues and developmental stages. In addition, *YUCCA*-mediated local auxin biosynthesis is widely involved in the regulation of vegetative growth and organ formation, affecting root development, plant morphogenesis, and vascular tissue differentiation, thereby highlighting its central role in the auxin metabolic network of plants [14–17].

Auxin plays an indispensable regulatory role in anther and pollen development. In *Arabidopsis thaliana* (*A. thaliana*), mutants affecting auxin signaling or metabolism related to reproductive development often exhibited severe abnormalities in floral organ development and male sterility [16,18]. Within the auxin biosynthesis pathway, *YUCCA2* and *YUCCA6* are the predominantly expressed *YUCCA* genes during pollen development, and the *yucca2 yucca6* double mutant fails to produce physiologically functional mature pollen, while other vegetative growth processes of the plant remain largely unaffected [19–21]. These findings clearly indicate that *YUCCA*-mediated local auxin biosynthesis constitutes a critical regulatory mechanism required for normal anther and pollen development in *A. thaliana*, and suggest that this regulatory module may be conserved across different plants.

The *YUCCA* gene family is highly conserved across the plant kingdom and has been systematically identified and functionally characterized in various model plants and important crops, including *A. thaliana*, rice (*Oryza sativa*), maize (*Zea mays*), barley (*Hordeum vulgare*), and soybean (*Glycine max*) [22]. In rice, *YUCCA*-mediated auxin biosynthesis participated in the regulation of crown root initiation and development, forming a regulatory model centered on YUC–IAA–WOX11 [23]. In soybean, several *YUCCA* genes are highly expressed in floral organs, and functional alterations of these genes significantly affect plant morphology and reproductive traits [24]. In maize, SPI1, which encodes a flavin-containing monooxygenase highly homologous to *A. thaliana* *YUCCA* proteins, exhibited clear defects in reproductive development when mutated, including reduced branching, fewer spikelets, and a lower seed set [25]. Moreover, in angiosperms, the asymmetric distribution of auxin during floral organ and gametophyte development mainly relies on *YUCCA*-mediated local biosynthesis rather than long-distance auxin transport [26].

Although previous studies have revealed the critical roles of the *YUCCA* gene family in auxin biosynthesis and the regulation of reproductive development across multiple plant species, the systematic composition, expression divergence, and specific functions of *YUCCA* genes in anther and pollen development remain largely unexplored in wheat due to its highly complex genome, abundant homologous gene copies, and significant functional redundancy. Therefore, systematic identification of the wheat *YUCCA* gene family, coupled with expression profiling to assess their relationship with pollen development and male fertility, is essential for elucidating the molecular mechanisms underlying auxin-mediated male reproductive development in wheat. Previous studies have identified 63 *YUCCA* genes in wheat using BLASTP, and suggested their potential roles in responses to abiotic stress [27]. However, the relationship between wheat *YUCCA* genes and male fertility has not been investigated. In this study, we combined HMMER and BLASTP to perform a genome-wide identification of the *TaYUCCA* gene family, resulting in 64 identified members. To further explore the evolutionary history of the *TaYUCCA* family, we also identified 28,

33, and 16 *YUCCA* genes in its closely related species *Aegilops tauschii* (*Ae. tauschii*), *Triticum dicoccoides* (*T. dicoccoides*), and *Triticum urartu* (*T. urartu*), respectively. Subsequently, we conducted comprehensive analyses of phylogeny, gene structure, and gene duplication events to characterize the evolution and structural features of the *TaYUCCA* gene family. In addition, promoter analysis, Gene Ontology enrichment, and expression profiling were performed to predict the potential functions of *TaYUCCA* genes. Finally, to investigate the roles of *TaYUCCA19* and its two homologs in wheat pollen development, we validated their functions using barley stripe mosaic virus-mediated virus-induced gene silencing (BSMV-VIGS).

2. Results

2.1. Genome-Wide Identification of the *YUCCA* Genes in Wheat and Its Three Progenitor Species

Both HMMER and BLASTP approaches were utilized to identify members of the *YUCCA* gene family in wheat and its progenitor species. A total of 64 *YUCCA* genes were identified in wheat, and the same strategy was used to identify 28, 33, and 16 *YUCCA* genes in *Ae. tauschii*, *T. dicoccoides* and *T. urartu*, respectively. The candidates were validated using InterPro and NCBI-CDD to confirm the conserved domains. All the genes were named based on their chromosomal locations within each species as follows: *TaYUCCA01-TaYUCCA64*, *AetYUCCA01-AetYUCCA28*, *TdYUCCA01-TdYUCCA33* and *TuYUCCA01-TuYUCCA16* (Table S1). Chromosomal mapping revealed that 62 *TaYUCCA* genes were unevenly distributed across all 21 wheat chromosomes, whereas two genes (*TaYUCCA63* and *TaYUCCA64*) were located on unassembled scaffolds. Among them, 16, 21, and 25 *TaYUCCAs* were located in the A, B, and D subgenomes, respectively (Figure S1).

The subcellular localization of 64 *TaYUCCA* proteins were predicted using the DeepLoc 2.1 online server, and the MW and pI were calculated using the pepstats module in EMBOSS (Table S2). The *TaYUCCA* proteins exhibit a highly conserved localization pattern. Specifically, 38 *TaYUCCA* proteins are predominantly localized in the cytoplasm, 21 are predicted to localize to both the cytoplasm and the endoplasmic reticulum, and only three are localized exclusively to the endoplasmic reticulum. In addition, the remaining two *TaYUCCA* proteins are predicted to be localized to the peroxisome and the plasma membrane, respectively. The *TaYUCCA* proteins ranged from 382 to 551 amino acids long with an average of 472 amino acids, and their MW ranged from 41.54 to 62.09 kDa.

2.2. Phylogenetic and Evolutionary Analysis of the *YUCCA* Gene Family in Wheat

To evaluate the evolutionary relationships of *YUCCA* genes in wheat, rice, and *A. thaliana*, a neighbor-joining (NJ) phylogenetic tree was constructed based on the multiple sequence alignment of 64 *TaYUCCAs*, 11 *AtYUCCAs*, and 23 *OsYUCCAs* full-length protein sequences (Figure 1A). The 98 genes were grouped into three major clades. Only clade III contains *AtYUCCA* genes. Additionally, 26 pairs of paralogous genes were identified within the NJ tree, including two pairs from rice, three pairs from *A. thaliana*, and 21 pairs from wheat. Considering that common wheat is a hexaploid species, its genome naturally harbors a higher number of homoeologous copies. Therefore, these paralogs in wheat are more likely derived from whole-genome duplication (WGD) and subsequent gene duplication events rather than species-specific expansions.

The same approach was used to construct phylogenetic trees for wheat and its three progenitor species (Figure 1B). A total of 195 genes were grouped into three clades. We identified 23 orthologous gene pairs between wheat and *Ae. tauschii*, 19 orthologous pairs between wheat and *T. dicoccoides*, and three orthologous pairs between wheat and *T. urartu*. This indicates a closer phylogenetic relationship between *TaYUCCA* and *AetYUCCA* and *TdYUCCA*.

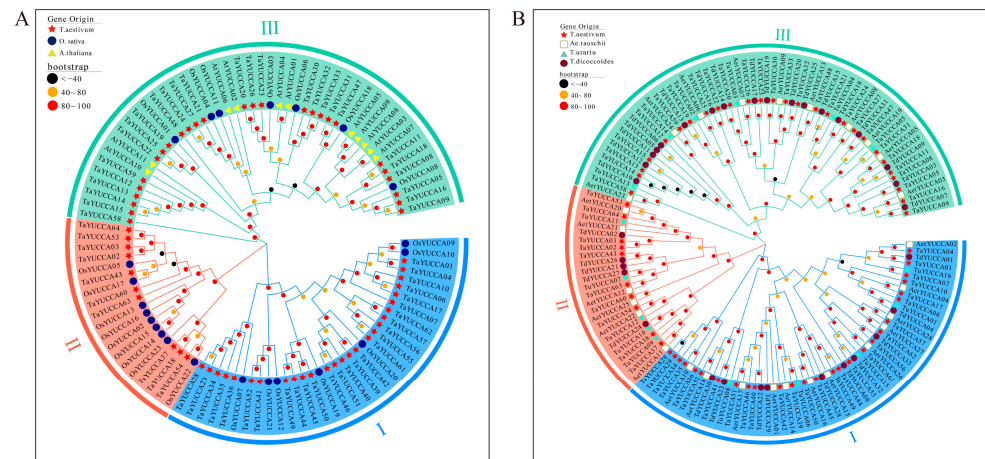


Figure 1. Phylogenetic analysis of the YUCCA gene family in wheat and other species. **(A)** Phylogenetic tree constructed using the full-length YUCCA protein sequences from *Triticum aestivum*, *A. thaliana*, and *Oryza sativa*. **(B)** Phylogenetic relationships of YUCCA genes between wheat and its progenitor species, including *Ae. tauschii*, *T. dicoccoides* and *T. urartu*. The trees were generated using the neighbor-joining method with 1000 bootstrap replicates. Different colored clades indicate distinct evolutionary groups.

2.3. Analyses of the Conserved Motifs and Gene Structure of the TaYUCCAs

Motif structures encoded by conserved genes and exon–intron organization represent important evolutionary signatures. A phylogenetic tree of the *TaYUCCAs* was constructed to elucidate the structural conservation of *TaYUCCAs* by dividing them into three clades (Figure 2). Overall, the members within the same clade exhibited similar conserved motifs and gene structures, whereas significant differences were observed among the motif number, arrangement, and exon count in the different clades. This indicates notable structural divergence within the gene family. Among the *TaYUCCAs*, Motif 2, Motif 18, Motif 3 and Motif 7 are ubiquitous core motifs and are present in all members. Moreover, substantial variation exists among the clades in terms of the size of clade and complexity of motif. Clade I and III are the largest clades, each containing 27 members. Each possessed more than 10 motifs. In contrast, Clade II contained only 10 members, and each gene harbors fewer than 10 motifs. Exon–intron analysis showed that more than 75% of *TaYUCCAs* contain four to five exons, whereas the remaining genes harbor only one to two exons, indicating that this gene family exhibits a high degree of structural conservation at the gene structure level during evolution. Together, these findings suggest that the *TaYUCCA* gene family has substantially diverged in their structures during evolution. The highly conserved motifs maintain the fundamental biochemical activities of the family, while the pronounced differences in gene structure and motif composition among clades drive the functional diversity of the *TaYUCCAs*.

2.4. TaYUCCAs Gene Duplication Events and Synteny Analysis

Gene duplication is a key driving force for the expansion and functional diversification of gene families. This study conducted a genome-wide identification of duplication events and analyzed the synteny to elucidate the evolutionary forces of the *TaYUCCA* family. A total of 36 duplicated gene pairs were identified among the *TaYUCCAs*, of which 28 pairs resulted from segmental duplication events and eight pairs from tandem duplication events (Figure 3A; Table S3). This indicates that segmental duplication is the predominant mechanism that drove the expansion of this gene family. To further explore the influence of natural selection on the *TaYUCCA* genes, the ratios of nonsynonymous (K_a) to synonymous (K_s) substitution rates were calculated. Based on alignments of CDS and protein sequences,

we found that 35 out of 36 (97.2%) identified duplicated gene pairs exhibited Ka/Ks ratios ranging from 0.03 to 0.60 (mean = 0.21), providing concrete evidence that the *TaYUCCA* gene family has undergone stringent purifying selection during its evolution from diploid ancestors to hexaploid wheat. Notably, while one gene pair exhibited a Ka/Ks ratio slightly above 1 (suggesting potential localized adaptive evolution), the vast majority of the family members are constrained by strong purifying selection to maintain their essential role in auxin biosynthesis. The use of the Ks values in conjunction with a molecular clock model enabled an estimation of the divergence time of these duplicated gene pairs of approximately 15.64 million years ago (Mya) on average.

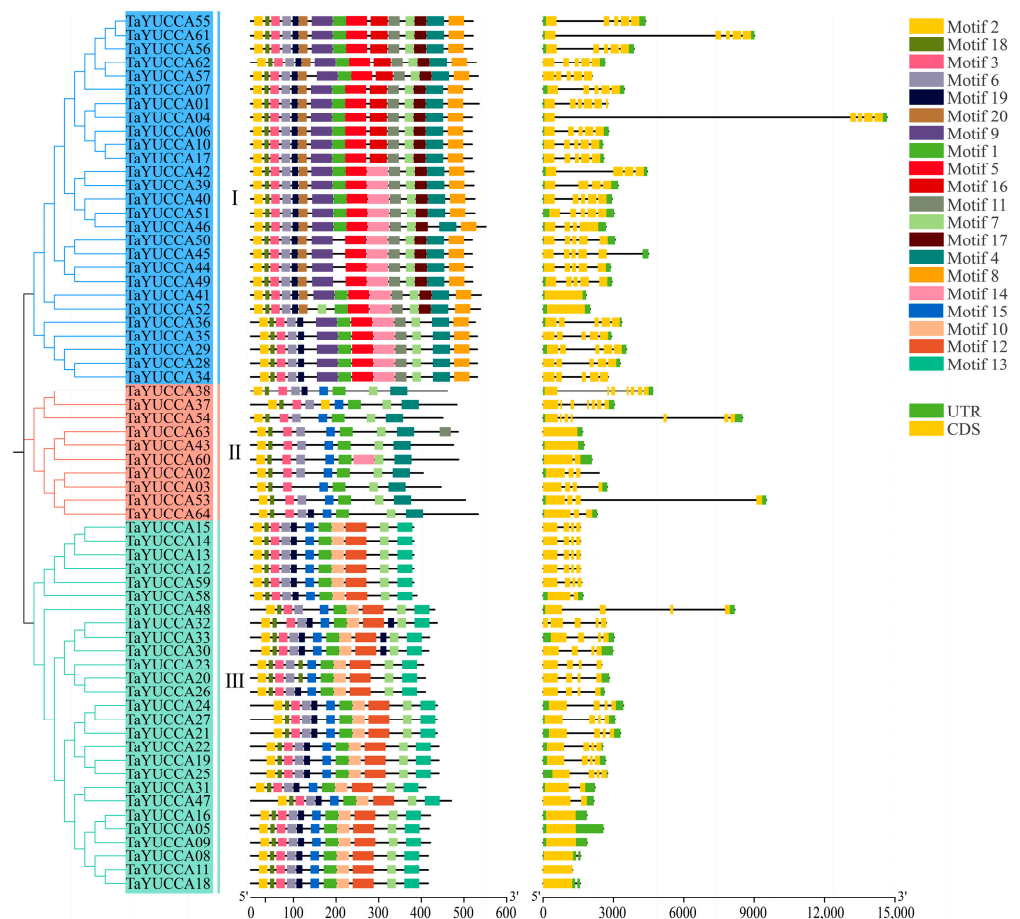


Figure 2. Phylogenetic relationships, conserved motifs, and gene structures of *TaYUCCAs*. The phylogenetic tree of *TaYUCCA* proteins is shown on the left. The conserved motifs of *TaYUCCA* proteins identified by MEME are displayed in the middle, with different colored boxes representing different motifs. The gene structures are shown on the right, where yellow boxes indicate CDS, black lines represent introns, and green boxes denote untranslated regions (UTRs).

The phylogenetic history of the *YUCCA* gene family was determined by conducting synteny analyses among common wheat and its three progenitor species, as well as rice (Figure 3B–E). A total of 31, 45, 15, and 19 duplicated gene pairs were identified in these comparative analyses (Tables S4–S7). The Ka/Ks ratios of all the duplicated gene pairs were <1 (ranging from 0.02 to 0.81 with an average of 0.25). This provided additional evidence that the *YUCCA* gene family has experienced strong purifying selection throughout its long evolutionary process from the diploid ancestors to the hexaploid common wheat, as well as the more distantly related rice, thereby maintaining the functional conservation of this gene family. The analysis of the time of divergence of syntenic gene pairs revealed the evolutionary history of the *YUCCA* gene family in the Poaceae species. The results

showed that the times of divergence between wheat and *Ae. tauschii*, *T. dicoccoides*, *T. urartu* were highly similar and estimated at approximately 6.16, 5.48, and 6.03 Mya, respectively. In contrast, the time of divergence between common wheat and rice was estimated at approximately 59.92 Mya. These findings suggest that the evolutionary timing of the *YUCCA* gene family is highly consistent with the phylogenetic relationships among these species. The divergence event between common wheat and rice has the oldest time of divergence, whereas the divergences between wheat and its progenitor species occurred more recently.

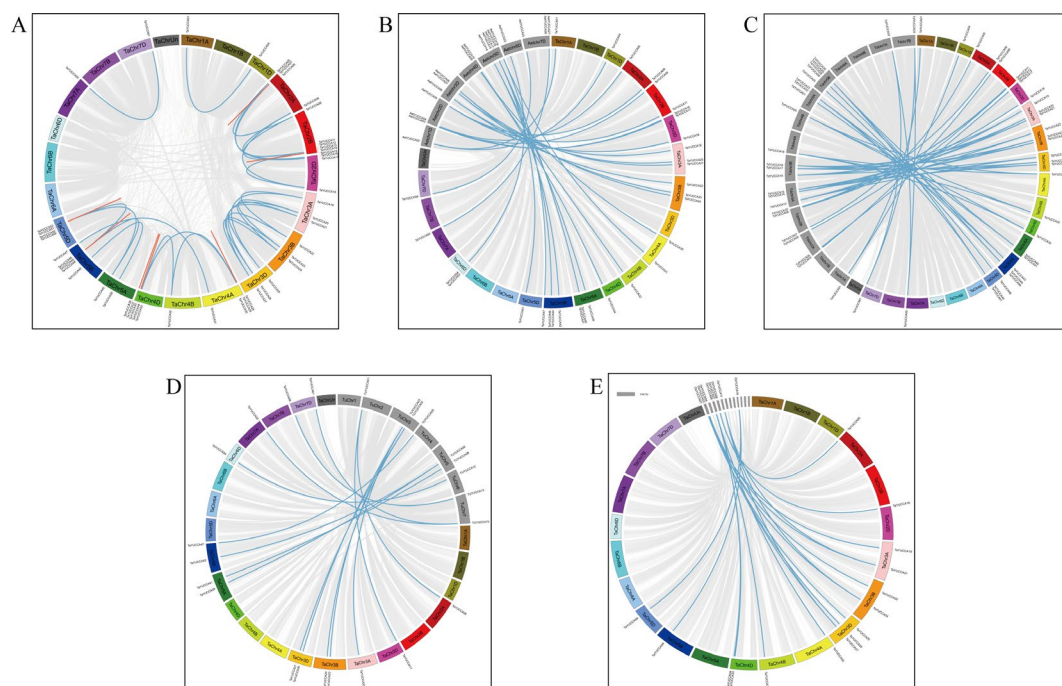


Figure 3. Synteny analysis of *TaYUCCA* genes in wheat and related species. (A) Syntentic relationships of *YUCCA* genes within the wheat genome. (B–E) Synteny analysis between wheat and related species, including *Ae. tauschii* (B), *T. dicoccoides* (C), *T. urartu* (D), and *Oryza sativa* (E). Chromosomes are shown in different colors. Blue lines represent segmentally duplicated *YUCCA* gene pairs, red lines indicate tandemly duplicated genes, and gray lines show syntenic blocks connecting the corresponding chromosomes.

2.5. Analysis of the *TaYUCCA* Gene Promoters

The *cis*-acting elements located within gene promoter regions are key regulatory structures that control the expression of genes. To identify such elements, the 2000 bp upstream sequences of *TaYUCCA* genes were extracted as putative promoter regions. They were then submitted to the PlantCARE database for an analysis of the *cis*-elements (Figure S2, Table S8). A total of 22 specific *cis*-acting elements were identified, which were classified into the following five major categories: hormone-responsive elements, stress-responsive elements, light-responsive elements, tissue-specific elements, and transcription factor-related *cis*-acting elements. The hormone-responsive elements accounted for approximately 37% of the total, including methyl jasmonate (MeJA)-responsive elements, abscisic acid (ABA)-responsive elements, salicylic acid (SA)-responsive elements, auxin-responsive elements, gibberellin-responsive elements and ethylene-responsive element. Notably, the MeJA-responsive elements were the most abundant and present in multiple copies in almost all the *TaYUCCA* gene promoters. Additionally, the other four hormone-responsive elements were widely distributed and occurred in more than 50% of the *TaYUCCA* promoter regions. Light-responsive elements were also present in multiple copies across all the *TaYUCCA*

promoters, including elements, such as the G-box and Sp1. These findings suggest that hormone-responsive and light-responsive elements are highly conserved and represent the two most important classes of *cis*-elements that regulate the transcription of *TaYUCCA*. Furthermore, several genes contained five types of environmental stress-responsive elements, including elements responsive to defense and stress, those responsive to low temperature, and a MYB binding site involved in the inducibility of drought, essential for the anaerobic induction element and wound-responsive elements. These findings suggest that *TaYUCCA* may play a significant role in the response to abiotic stress. Two types of tissue development-specific elements—endosperm expression regulatory elements, meristem expression elements and seed-specific regulatory elements—were also present in the promoters of most genes. This indicates that the *TaYUCCA* genes play indispensable roles at multiple stages of plant development. MYB binding sites are widely present in *TaYUCCA* promoters, suggesting that MYB transcription factors may serve as conserved regulators of *YUCCA* gene expression.

2.6. Gene Ontology (GO) Annotation Analysis of the *TaYUCCAs*

The functional characteristics of the *TaYUCCA* genes were analyzed in more detail by conducting a GO annotation for all 64 *TaYUCCAs* (Figure 4; Table S9). The results showed that all genes were categorized into 17 GO terms within the biological process and molecular function categories. Five GO terms are closely associated with auxin biosynthesis, with nearly all *TaYUCCA* genes annotated to the NADP binding and N,N-dimethylaniline monooxygenase activity terms, encompassing 64 and 59 genes, respectively. This observation is highly consistent with the pivotal role of *TaYUCCA* genes in the biosynthesis of auxin. Seven GO terms are related to plant immunity or disease resistance, each comprising the same 27 genes, indicating that all these genes share highly conserved functions and play key roles in plant stress responses. The remaining four GO terms are closely associated with plant growth and development. Notably, *TaYUCCA19*, *TaYUCCA22*, and *TaYUCCA25* are enriched in the anther dehiscence term, suggesting that these genes are indispensable for anther development.

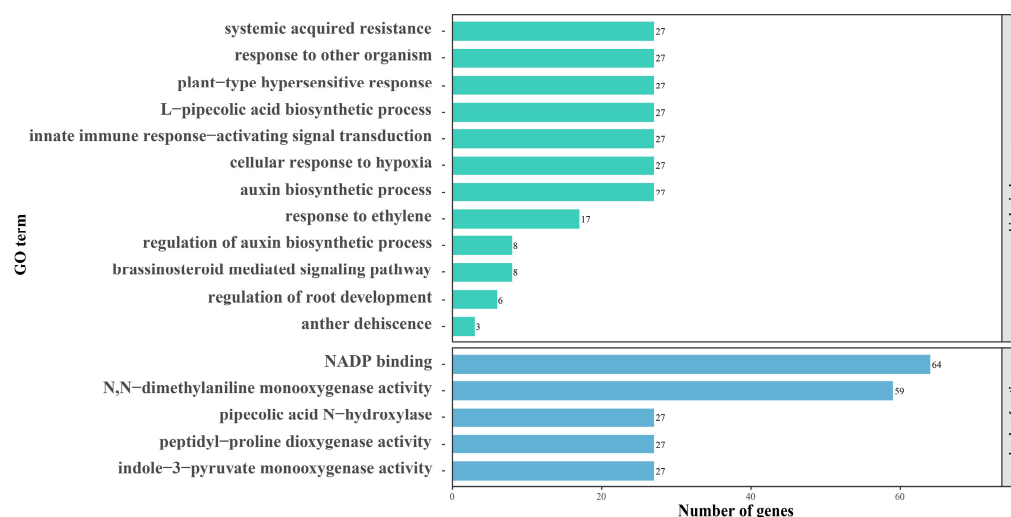


Figure 4. Gene Ontology (GO) annotation of *YUCCAs*. *YUCCAs* were annotated with GO terms classified into biological process (BP) and molecular function (MF) categories. The x-axis shows the number of genes annotated to each GO term, and the y-axis lists the corresponding GO terms.

2.7. Expression Patterns Analysis of the *TaYUCCAs* in Different Tissues

Based on the Transcripts Per Million (TPM) values obtained from the WheatOmics database, we analyzed the tissue-specific expression patterns of *TaYUCCAs* in roots, stems,

leaves, spikes, and grains of wheat (Figure 5A). The results revealed a substantial divergence in expression among the five tissues. A total of 34 genes were specifically and highly expressed in the roots, this suggests that these genes may function specifically in the root. 12 genes were highly expressed in the stems, which suggests that these *TaYUCCAs* may play more critical roles in wheat stem development compared to other family members. Only four *TaYUCCA* genes show relatively high expression in leaves. Nine genes are highly expressed in spikes, including four *TaYUCCA* genes that are specifically highly expressed in spikes. The remaining five genes not only show high expression in spikes but also maintain relatively elevated expression levels in grains. Only six genes were highly expressed in the grains, which suggests that these *TaYUCCAs* may play more critical roles in grain development and accumulation of energy than the other family members. The distinct preferential expression of the different *TaYUCCAs* in particular tissues further supports the functional diversification within this gene family. In addition, the dynamic expression of the *TaYUCCAs* exhibited pronounced variation across the four stages of wheat meiosis (Figure 5B). Only seven *TaYUCCAs* were highly expressed in metaphase I, which suggests that these genes may predominantly function at this stage. Five *TaYUCCAs* were markedly highly expressed at the latent_lepto stage but maintained relatively low levels of expression thereafter, which suggests their potential importance in initiating meiosis. Notably, more than 15 genes showed high levels of expression during two critical stages of meiosis (diplo_dia and zygo_pachy). This stage-specific high level of expression indicates that the *YUCCA* genes play an important role in the core biological process of meiosis.

2.8. Expression Patterns Analysis of the *TaYUCCAs* in Anthers

The relationship between *TaYUCCAs* and fertility conversion was explored in the thermo-sensitive male sterile wheat line YS3038 by examining the expression patterns of 16 *TaYUCCAs* using qRT-PCR in anthers at the uninucleate (U), binucleate (B), and trinucleate (T) stages under both fertile (YSF) and sterile (YSS) conditions (Figure 6A; Table S10). Among the 10 genes specifically highly expressed at the fertile trinuclear stage, three genes (*TaYUCCA31*, *TaYUCCA26*, and *TaYUCCA54*) exhibit low expression under sterile conditions at the same stage. *TaYUCCA46*, *TaYUCCA51*, and *TaYUCCA40* show a continuously decreasing expression trend during pollen development under both fertile and sterile conditions, indicating that these three genes play important roles in the early stages of pollen development. A total of nine *TaYUCCA* genes (*TaYUCCA19*, *TaYUCCA22*, *TaYUCCA46*, *TaYUCCA51*, *TaYUCCA40*, *TaYUCCA25*, *TaYUCCA39*, *TaYUCCA05*, and *TaYUCCA27*) display significantly higher expression levels at the fertile uninuclear stage compared with the corresponding sterile conditions. Notably, *TaYUCCA19*, *TaYUCCA22*, and *TaYUCCA25* exhibit distinctly different expression patterns between fertile and sterile conditions across the uninuclear, binuclear, and trinuclear stages. Compared with fertile conditions, these genes show significantly reduced expression at the uninuclear stage under sterile conditions; their expression is upregulated at the binuclear stage, but the difference between fertile and sterile conditions is not significant. At the trinuclear stage, these three genes maintain relatively high expression under sterile conditions, whereas their expression under fertile conditions has markedly declined to low levels (Figure 6B). Transcriptomic data (unpublished) similarly support these expression patterns (Figure S3), indicating that *TaYUCCA19*, *TaYUCCA22*, and *TaYUCCA25* may play critical roles in anther development, particularly during the uninuclear stage.

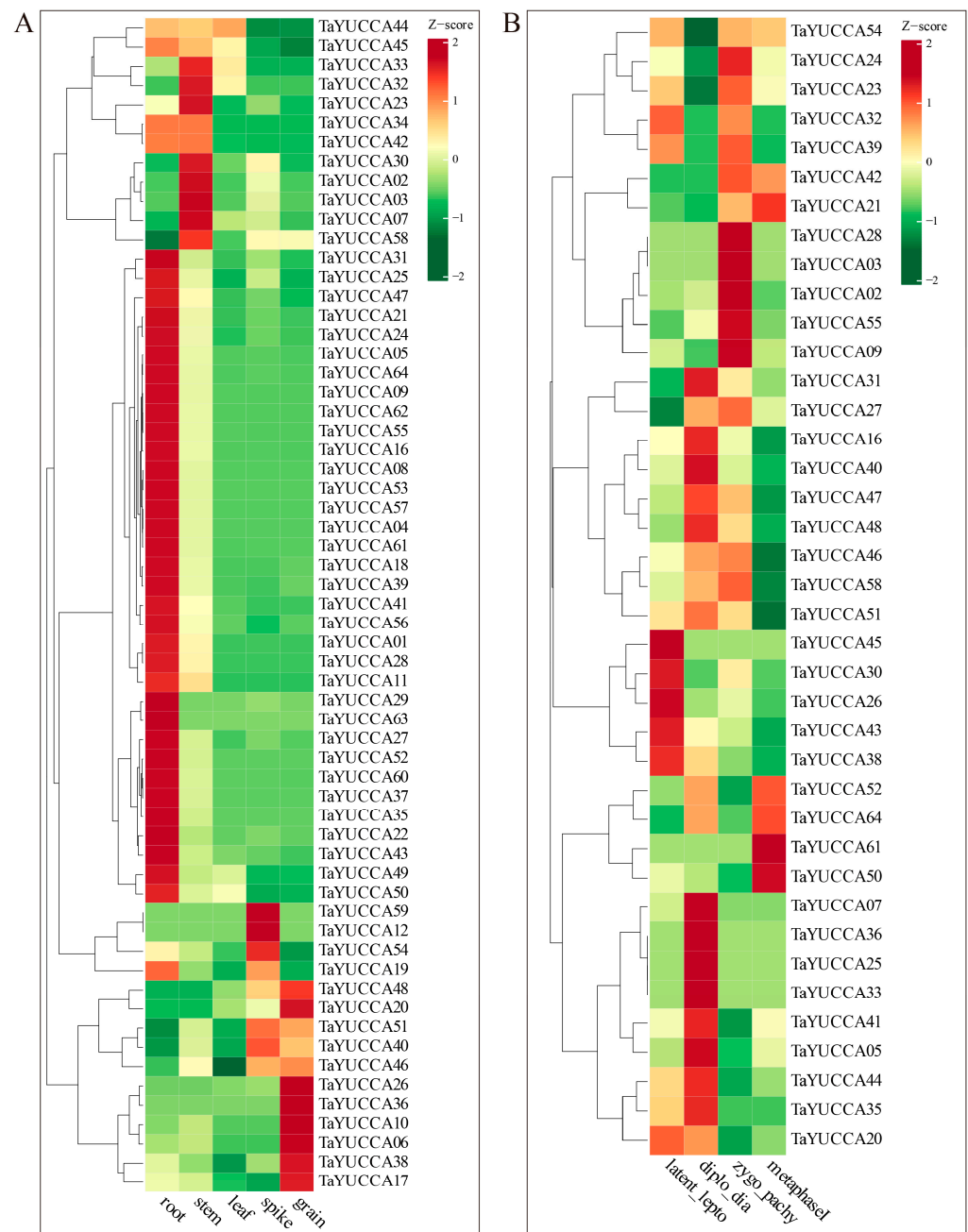


Figure 5. Expression patterns of *TaYUCCAs* in different tissues and meiotic stages. **(A)** Heatmap showing *TaYUCCAs* expression levels in five wheat tissues: root, stem, leaf, spike, and grain. **(B)** Heatmap illustrating expression patterns of *TaYUCCAs* during four meiotic stages: latent_lepto, diplo_dia, zygo_pachy, and metaphase I. Expression values were normalized using Z-score transformation.

2.9. Roles of *TaYUCCA19* and Its Homologous Genes in Wheat Pollen Development

To investigate the role of *YUCCA* genes in male reproductive development in wheat, barley stripe mosaic virus-mediated virus-induced gene silencing (BSMV-VIGS) was employed to analyze function. Four treatment groups exhibited corresponding phenotypes after infection (Figure S4). The leaves of the control group had no stripes, which would be a symptom of disease. In contrast, the other three groups developed typical striped lesions, and the leaves of the positive control bleached. These results confirmed the efficacy of the VIGS system in this experiment.

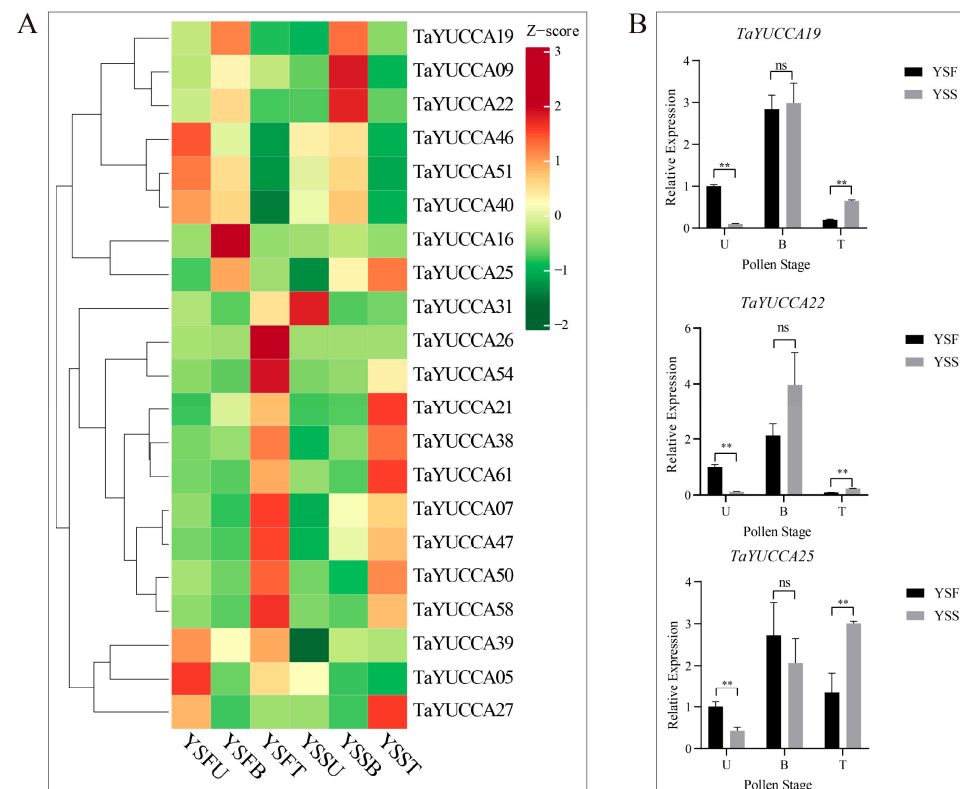


Figure 6. Expression of *TaYUCCAs* in wheat anthers under fertile and sterile conditions by qRT-PCR. (A) Heatmap showing relative expression levels of *TaYUCCAs* across different pollen developmental stages in fertile and sterile anthers. (B) Relative expression levels of *TaYUCCA19*, *TaYUCCA22* and *TaYUCCA25* at various pollen developmental stages. The x-axis represents pollen developmental stages, and the y-axis shows relative expression levels. Black bars indicate fertile (YSF) samples, while gray bars indicate sterile (YSS) samples. “**” denote statistically significant differences between fertile and sterile conditions (Student’s *t*-test, $p \leq 0.01$).

At the trinucleate stage of anther development, the anthers from the negative control and *TaYUCCA19*-silenced plants were stained with iodine-potassium iodide (I_2 -KI) (Figure 7A,B). Microscopic observations showed that pollen grains from the negative control plants were uniformly stained, regularly shaped, and structurally intact, whereas the pollen grains in the gene-silenced plants stained less intensely, had an irregular morphology, and partially collapsed. Pollen counting showed that the negative control contained a total of 263 pollen grains, and 2 of them were lightly stained or morphologically abnormal. This corresponded to an abnormal pollen rate of 0.76%. In contrast, the gene-silenced plants produced 310 pollen grains in total, and 307 were abnormal, corresponding to an abnormal pollen rate of 99.03% (Figure 7C). These results indicate that the development of pollen was severely impaired in the gene-silenced plants, with a significantly higher proportion of abnormal pollen compared with the negative control. Subsequently, the expression level of *TaYUCCA19* in the anthers at the uninucleate stage was examined in both treatments (Figure 7D). The results showed that the expression of *TaYUCCA19* was significantly reduced in the silenced plants compared with the negative control. Notably, *TaYUCCA19* has a highly similar sequence to its two homologs, *TaYUCCA22* and *TaYUCCA25*, with an overall sequence identity of 98.27%; the identity within the VIGS-targeted region reached 97.86% (Figure S5). Therefore, the expression levels of *TaYUCCA22* and *TaYUCCA25* were also significantly reduced (Figure 7D). A significant difference in the seed-setting rate was observed between the two treatments. Ten spikes were examined and analyzed statistically for each treatment (Figure 7E,F; Table S11). The negative control plants produced

relatively plump grains, with a seed-setting rate of approximately 88.68%, whereas the *TaYUCCA19*-silenced plants were almost completely sterile, showing a seed-setting rate of only 5.99%. Taken together, these results demonstrate that *TaYUCCA19* and its homologous genes are indispensable for the development of normal pollen and the maintenance of fertility in wheat.

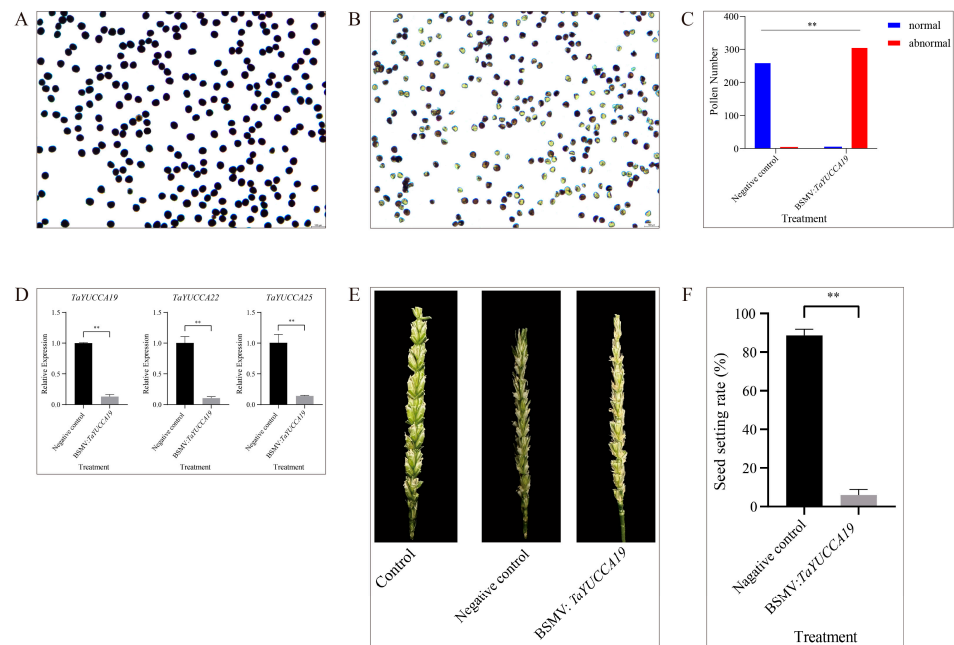


Figure 7. Effects of *TaYUCCA19* silencing on pollen development, seed setting, and gene expression in wheat. Effects of *TaYUCCA19* silencing on pollen development, seed setting, and gene expression in wheat. (A,B) I2-KI staining of pollen grains from negative control (A) and *TaYUCCA19*-silenced plants (B). (C) Quantification of pollen viability. blue bars represent normal pollen, while red bars represent abnormal pollen. Statistical significance was determined using Fisher's exact test. ** indicates $p \leq 0.01$. (D) qRT-PCR analysis of *TaYUCCA19*, *TaYUCCA22*, and *TaYUCCA25* expression in uninucleate-stage anthers of negative control and *TaYUCCA19*-silenced plants. (E) Representative spike phenotypes showing fertility of negative control and *TaYUCCA19*-silenced plants. (F) Seed set rate of negative control and *TaYUCCA19*-silenced plants. "***" indicates statistically significant differences (Student's *t*-test, $p \leq 0.01$).

3. Discussion

The core feature of the *YUCCA* gene family is that all its members contain the Flavin-binding monooxygenase-like (PF00743). The *YUCCA* family is essential for auxin biosynthesis in plants [28]. This gene family has been identified in maize, *A. thaliana*, and *Brassica rapa*, as well as in other plant species [19,24,29]. Although previous studies have investigated the *YUCCA* gene family in wheat, differences in the identification methods have led to inconsistencies in the members reported [27]. In this study, we utilized a combined strategy using HMMER and BLASTP to identify 64 *TaYUCCAs* in wheat. Additionally, 28 *AetYUCCAs*, 33 *TdYUCCAs* and 16 *TuYUCCAs* were identified in its progenitor species. The number of *YUCCA* genes in wheat is significantly higher than that in its diploid and tetraploid progenitors. There was a gradual increase with increasing ploidy level, which is highly consistent with the evolutionary history of wheat. The *YUCCA* gene family markedly expanded during the evolution of wheat. This expansion was primarily driven by whole-genome duplication (WGD) events. The high retention rate of the family members following WGD suggests that this gene family plays important roles in the growth and development of wheat. Notably, this pattern of expansion is consistent with previous observations in wheat gene families, such as the *E-class* [30] and *TPS* [31], whose copy numbers also increase

with ploidy level, which further confirms the central role of WGD in the expansion of gene families in wheat. Compared with diploid species such as *A. thaliana*, rice and maize, wheat possesses the largest number of *YUCCA* family members. This is primarily because wheat is an allohexaploid (AABBDD) species. During WGD, it retained a large number of homologous genes from its progenitor species, and subsequent gene duplication events further promoted the expansion of the *YUCCA* gene family in wheat [32]. Significant variation was observed in the length of amino acids, MW, pI, and subcellular localization among the TaYUCCA proteins. This indicated that while the conserved domains are maintained, functional divergence has occurred among the family members to some extent [33].

The phylogenetic relationships of the wheat *YUCCA* gene family were comprehensively analyzed by constructing three types of phylogenetic trees. The first was an intra-species phylogenetic tree containing only wheat *YUCCA* family members, which was constructed to elucidate the internal structure and characteristics of the classification of this family within wheat. Secondly, a cross-species phylogenetic tree based on *YUCCA* protein sequences from wheat, maize, and *A. thaliana* was generated to compare the phylogenetic relationships and evolutionary divergence patterns of the *YUCCA* genes between monocotyledonous and dicotyledonous plants. Third, a phylogenetic tree that integrated the *YUCCA* protein sequences from wheat and its progenitor species was constructed to trace the evolutionary origins and trajectories of the TaYUCCA gene family during the evolution of wheat. This multi-dimensional phylogenetic analysis provides a solid foundation to understand the expansion, divergence, and conservation of the *YUCCA* genes in wheat [34]. The phylogenetic analysis revealed that all the trees could be divided into three clades. All AtYUCCA genes are clustered within clade III, a distribution pattern that differs from the *YUCCA* gene family organization reported in *Vigna radiata* L. and *Glycine max* L. [35,36]. This discrepancy may reflect evolutionary divergence of the *YUCCA* gene family between monocot plants (particularly wheat) and dicot plants. In the phylogenetic tree that involves wheat and its progenitor species, all three clades contained *YUCCA* genes from all five species. This indicates that the family is largely intact across these lineages without obvious gene loss. Gene structure analysis further revealed differences among the clades. Although the motifs present in the genes of the three clades exhibit specific characteristics, the overall gene structures are relatively conserved. This suggests that, despite certain structural differences between clades, the structural features within each clade are highly conserved, reflecting that the organizational patterns of the *YUCCA* gene family have remained conserved throughout long-term evolution.

In the phylogenetic tree constructed from wheat and its four progenitor species, the *YUCCA* gene family exhibits typical differentiation corresponding to the A, B, and D genomes. Orthologous gene pairs were identified as 23 and 19 between wheat and *Ae. tauschii* (DD) and *T. dicoccoides* (AABB), respectively, indicating a high degree of inheritance and the retention of *YUCCA* genes in these genomes. This distribution pattern is highly consistent with the evolutionary history of common wheat: *Ae. tauschii*, as the direct donor of the D genome, shares the shortest evolutionary distance with wheat, thus, retaining more one-to-one homologous relationships within the *YUCCA* family. *T. dicoccoides* as the primary donor of the A and B genomes in modern wheat, also maintain high sequence similarity and phylogenetic clustering relationships. In contrast, only three orthologous gene pairs were detected between wheat and *T. urartu* (AA). This phenomenon may be attributed to two reasons. First, the A genome of common wheat evolved through two rounds of distant hybridization [37], during which complex genome reshaping, gene duplication, and structural variations probably occurred. These changes resulted in *YUCCA* genes derived from the A subgenome that was more distinct from those of *T. urartu*. Secondly, in addition to WGD, the TaYUCCAs may also have been influenced by local duplication events, such as

segmental duplication and transposon-mediated duplication during evolution, which led to the redistribution of some gene copies within the genome and weakened their homologous relationships with the *TaYUCCAs*.

Duplicated genes are widespread in plant genomes and play an essential role in the diversification of new functions, such as the development of floral organs and the adaptation to biotic and abiotic stresses [38]. Gene duplication is a major driving force for the expansion of gene families and the emergence of novel gene functions [39], which primarily occurs through tandem duplication, segmental duplication, transposition events, and WGD [40–43]. This study systematically analyzed the duplication events of *YUCCA* genes within wheat and further compared the duplication and conservation patterns between wheat and its three progenitor species, as well as rice, to elucidate the expansion mechanisms of the *YUCCA* gene family. A total of 36 duplicated gene pairs were identified within wheat, of which 28 pairs involved segmental duplication and 8 pairs involved tandem duplication. A total of 31, 45, 15, and 19 duplicated gene pairs were detected between wheat and each of its three progenitor species and rice, respectively. All of these events involved segmental duplication. This indicated that in addition to the whole-genome duplication, local segmental duplication also substantially contributed to the further expansion of the *YUCCA* gene family in wheat. Previous studies have demonstrated that segmental duplication and chromosomal translocation facilitate rapid environmental adaptation in plants [44]. Therefore, this feature may partly reflect the adaptive potential of common wheat during long-term evolution and provide important clues to understand the molecular basis that underlies its broad environmental adaptability.

The K_a/K_s ratio is a widely used parameter in evolutionary studies, and it reflects the rates of nonsynonymous (K_a) and synonymous (K_s) substitutions and thus, provides insights into the selective pressures that act on genes. Generally, $K_a/K_s > 1$ indicates positive selection, whereas $K_a/K_s < 1$ suggests purifying selection [45]. In addition, divergence time can be estimated based on the K_s values [46]. In this study, all duplicated gene pairs exhibited $K_a/K_s < 1$, suggesting that the *YUCCA* gene family has experienced strong purifying selection throughout the evolutionary history from diploid progenitor species to hexaploid common wheat, as well as during its divergence from the distantly related species rice, thereby maintaining structural and functional conservation of these genes [47]. Furthermore, the estimation of the divergence time revealed that the divergence between wheat and its progenitor species occurred much more recently than that between wheat and rice, which is highly consistent with the phylogenetic relationships among the plants and the general evolutionary pattern of species divergence.

Variations in the types and distributions of *cis*-acting elements in promoter regions are important contributors to the diversification of gene regulation and functional differentiation [48]. In the promoter regions of all the *TaYUCCAs*, we identified five major categories of characteristic *cis*-acting elements, including elements responsive to hormones, environmental stress, light and *cis*-acting elements related to transcription factors. In addition, there are specific elements that respond to tissues and development. Elements that respond to MeJA and ABA were widely detected in the promoters of the *TaYUCCAs*, which suggests that these hormones may participate in the transcriptional regulation of *TaYUCCAs*. Similarly, multiple elements that respond to hormones have also been identified in the *YUCCA* gene family of Japanese apricot (*prunus mume*) [49]. In addition, all *TaYUCCA* promoters contained at least one *cis*-acting element associated with the responses to abiotic stress, suggesting that these elements may regulate the expression of *YUCCA* genes under stress conditions and thereby enhance plant adaptability to adverse environments. In plants, *YUCCA* genes have been shown to participate in responses to abiotic stresses such as drought [50] and heat [51]. MYB-binding sites are widely distributed in the

promoter regions of *TaYUCCA* genes. Numerous studies have demonstrated that MYB family transcription factors are closely associated with pollen development in plants [52–54]. Accordingly, we propose that *TaYUCCA* genes may be regulated by MYB transcription factors during anther development.

It is generally believed that gene function depends on its spatiotemporal expression characteristics, and the expression patterns of the genes across different tissues, developmental stages, and environmental conditions provide important evidence to understand their potential biological functions [55]. This study analyzed the expression levels of *TaYUCCAs* using transcriptome data from the WheatOmics database. This facilitated the analysis of their levels of expression in the wheat roots, stems, leaves, spikes, and grains, as well as in the anthers at four meiotic stages. The results showed that individual *TaYUCCAs* exhibited distinct expression patterns among different tissues and developmental stages. However, several gene clusters were only highly expressed in certain tissues or at specific developmental stages, suggesting that the different *TaYUCCAs* may perform diverse biological functions. Genes that were closely clustered in the phylogenetic tree tended to show similar expression patterns, such as *TaYUCCA21* and *TaYUCCA24*, which were highly expressed in leaves, and *TaYUCCA06*, *TaYUCCA10*, and *TaYUCCA14*, which were preferentially expressed in grains. This observation is consistent with previous findings that the duplicated genes generated by segmental duplication or WGD often display similar and functionally redundant expression patterns across multiple tissues [56]. However, during the four stages of meiosis, most homologous gene pairs exhibited markedly divergent expression patterns, indicating substantial expression divergence of *TaYUCCAs* during reproductive development in wheat. This suggests that they have a relatively low functional redundancy, and they may not exhibit overlapping functions across all stages of development.

Previous studies have demonstrated that the *YUCCA* genes in multiple species are closely associated with male fertility in plants. In *A. thaliana*, triple and quadruple mutants composed of *YUCCA1* and *YUCCA4*, and *YUCCA2* and *YUCCA6* exhibit severe defects in multiple developmental processes, including floral organ morphogenesis and vascular tissue formation. Among these, the *yuc2yuc6* and *yuc1yuc4* mutants largely display a sterile phenotype, and the *yuc2yuc6* mutant shows markedly delayed anther maturation, producing almost no pollen [19]. In barley, the *MSG38* gene encodes a *YUCCA*-type flavin monooxygenase involved in auxin biosynthesis, and the *msg38* mutant exhibits a complete male sterility. During anther maturation, the overall morphological development of *msg38* anthers is largely normal; however, at the anther dehiscence stage, the anthers fail to turn yellow and dehisce properly due to delayed or failed separation of the specialized septum and stomium cells, preventing pollen release. In addition, although the pollen of *msg38* mutants appears viable, its size is significantly reduced, and its staining response to I₂-KI is markedly weakened [57]. In maize, seven *YUCCA* genes, including *ZmYUCCA2*, are predominantly expressed in reproductive organs, with *ZmYUCCA9* and *ZmYUCCA10* showing high expression in the tassel and anthers. mRNA in situ hybridization revealed that *ZmYUCCA10* is specifically expressed in the tapetum, microspores, and mature pollen, suggesting that it may play a key role in pollen development [29].

Even though the *YUCCA* genes in multiple species have been verified to be associated with male sterility, studies on the relationship between *YUCCA* genes and male fertility in wheat, a polyploid crop, remain limited. In addition, the highly homologous members of the *YUCCA* gene family may exhibit functional redundancy. Therefore, a systematic analysis of the wheat *YUCCA* gene family, investigation of their expression patterns, and integration with functional validation are highly important for elucidating their roles in the regulation of male fertility, optimizing the utilization of male-sterile lines, and guid-

ing hybrid breeding strategies. GO annotation analysis revealed that among the wheat *YUCCA* gene family members, only *TaYUCCA19*, *TaYUCCA22*, and *TaYUCCA25* were annotated in the anther dehiscence pathway, indicating that these three genes are critically important for pollen development. Moreover, in the temperature-sensitive male-sterile wheat line YS3038, the expression levels of these three genes were significantly reduced under sterile conditions at the uninucleate pollen stage. Following silencing of *TaYUCCA19* using BSMV-VIGS, the plants exhibited abnormal pollen morphology and a significantly decreased seed-setting rate. The principle of VIGS is to insert a partial sequence of the target gene into a viral vector, which is transcribed in host cells to generate double-stranded RNA (dsRNA). This dsRNA is subsequently processed by Dicer-like enzymes into short interfering RNAs (siRNAs) of approximately 21–23 nucleotides. These siRNAs are then incorporated into the RNA-induced silencing complex (RISC) and direct the degradation of transcripts that share at least 23 consecutive nucleotides of sequence identity with the target. Consequently, this technique inevitably silences homologous genes with high sequence similarity [58,59]. In this study, *TaYUCCA19* shows high sequence similarity with its two homologous genes, *TaYUCCA22* and *TaYUCCA25*. Therefore, the siRNAs derived from the *TaYUCCA19*-targeting fragment are able to recognize and act on the transcripts of these homologs as well, resulting in their concomitant down-regulation in silenced plants. To some extent, this coordinated silencing of homologous genes helps to reduce functional redundancy among *TaYUCCA19* and its homologs, thereby providing a more comprehensive understanding of their roles in wheat male reproductive development. In the barley *msg38/yucca4* mutant, pollen-localized auxin biosynthesis is downregulated, leading to the decreased expression of energy metabolism-related genes and preventing pollen from reaching a high-energy state. This suppresses glycolytic and TCA cycle fluxes, ultimately resulting in a marked reduction in starch accumulation, indicating that auxin drives pollen maturation by regulating central carbon metabolism [57]. Similarly, in *TaYUCCA19*-silenced plants, pollen grains stained weakly with I₂-KI, further suggesting reduced pollen viability and disrupted metabolic activity. In summary, these results suggest that *TaYUCCA19*, *TaYUCCA22* and *TaYUCCA25* play an important role in energy and metabolite regulation during pollen development and are indispensable for male reproductive development in wheat. Although this study preliminarily analyzed the potential roles of *TaYUCCA19* and its homologous genes in anther development using VIGS, this approach is inherently limited, because VIGS induces only transient gene silencing and cannot selectively suppress highly homologous genes, thereby constraining independent functional validation of individual genes. Moreover, the specific signaling pathways and metabolic processes regulated by *TaYUCCAs* during wheat development remain unclear. Therefore, systematic functional verification using stable mutant lines will be required to comprehensively elucidate the molecular mechanisms underlying the role of this gene in male reproductive.

4. Materials and Methods

4.1. Identification of *YUCCA* Gene Family Members in Wheat and Its Progenitor Species

The genome data of wheat and its progenitor species were obtained from the Ensembl Plants database (<https://plants.ensembl.org/index.html>, accessed on 9 August 2025). The Hidden Markov Model (HMM) profile of the Flavin-binding monooxygenase-like (FMO) domain (PF00743) was obtained from the InterPro database (<https://www.ebi.ac.uk/interpro/>). Using the hmmsearch program from the HMMER 3.3.2 software package, proteins containing this domain were identified from the local wheat protein database, with an initial E-value cutoff of 1×10^{-20} . Subsequently, the domain sequences of the candidate proteins were extracted and subjected to multiple sequence alignment using MUSCLE 5.1 [60]. Based on this alignment, a wheat-specific hidden Markov model was constructed

using the hmmbuild tool [61]. Finally, the resulting model was used for a second round of screening against the local wheat protein database using hmmsearch, with the E-value threshold set to 1×10^{-10} . Subsequently, the *YUCCA* genes of *A. thaliana* were obtained from the TAIR database (<https://www.arabidopsis.org/>) were used as queries. BLASTP searches (using BLAST 2.14.0) were performed against the local wheat protein database with an E-value threshold of 1×10^{-5} . Protein sequences obtained from both methods were integrated and manually deduplicated, and they yielded candidate members of the wheat *YUCCA* gene family. These candidates were submitted to InterPro [62] and NCBI-CDD to confirm the presence of the FMO domain, thereby determining the final *YUCCA* gene family members. Additionally, the *YUCCA* gene family members in the following progenitor species of wheat: *Ae. tauschii*, *T. dicoccoides* and *T. urartu* were identified using the same methods. The chromosomal physical localization information was sourced from the wheat genome annotation data (gff3), and MG2C was employed to map the wheat *YUCCA* gene family members to chromosomes [63]. The molecular weight (MW) and isoelectric point (pI) of the wheat *YUCCA* family proteins were calculated using the pepstats module of EMBOSS 6.6.0 [64]. Subcellular localization of the family members was predicted using the online tool DeepLoc 2.1 (<https://services.healthtech.dtu.dk/services/DeepLoc-2.1/>, accessed on 15 December 2025).

4.2. Phylogenetic Relationship, Gene Structure, and Conserved Motif Analysis

To investigate the evolutionary relationships of the wheat *YUCCA* gene family, protein sequences of *YUCCA* family members from wheat, rice, *A. thaliana*, *Ae. tauschii*, *T. dicoccoides* and *T. urartu* were aligned using the ClustalW program implemented in MEGA11 [65]. Subsequently, phylogenetic trees were constructed using the neighbor-joining (NJ) method with 1000 bootstrap replicates. The phylogenetic tree was visualized using Evolview [66].

Information about the gene structures of *TaYUCCAs* was extracted from genome annotation files to analyze the patterns of distribution of the introns and exons. Conserved motifs in the *TaYUCCA* protein sequences were identified using MEME 5.5.2 [67] with default parameters and a maximum motif number set to 20. The results were visualized using TBtools 2.423 [68].

4.3. Gene Duplication and Synteny Analysis

The evolutionary relationships and syntenic characteristics of the *YUCCA* gene family in wheat and its related species were elucidated by conducting gene duplication and synteny analyses among wheat, *Ae. tauschii*, *T. dicoccoides*, *T. urartu*, and rice. MCScanX [69] was used for the synteny analysis. Circos 0.69.9 [70] was used for visualization. The non-synonymous substitution rate (Ka) and synonymous substitution rate (Ks) were calculated then using the TBtools [68]. Finally, the divergence time (T) of the syntenic gene pairs was estimated using the formula, with $\lambda = 6.5 \times 10^{-9}$ for Gramineae [32].

$$T = Ks / (2\lambda) \times 10^6 \text{ Mya}$$

4.4. Promoter Analysis of the *TYUCCAs*

The 2000 bp upstream DNA sequences of *TaYUCCAs* were retrieved from the Ensembl Plants database and subsequently submitted to the PlantCARE database (<https://bioinformatics.psb.ugent.be/webtools/plantcare/html/>, accessed on 13 December 2025) to predict the *cis*-acting regulatory elements in the promoter regions.

4.5. Analysis of the GO (Gene Ontology) Annotation

All 64 *TaYUCCA* genes were submitted to the TGT database (<https://wheat.cau.edu.cn/TGT/>, accessed on 20 December 2025) for the GO annotation analysis, and the results were visualized using R 4.3.1.

4.6. Analysis of the Tissue Expression Patterns

The WheatOmics database [71] was the source of the transcriptome data of *TaYUCCAs* in five tissues (roots, stems, leaves, spikes, and grain) and at four meiotic stages, including the latent leptotene to leptotene (latent_lepto), diplotene to diakinesis (diplo_dia), zygotene to pachytene (zygo_pachy), and metaphase I. Expression heatmaps were constructed using R.

4.7. Plant Materials and qRT-PCR Analysis

The relationship between *TaYUCCAs* and pollen fertility in wheat was investigated using the temperature-sensitive male-sterile line YS3038, which was developed at the College of Agronomy, Northwest A&F University. The plants were vernalized in June 2025, cultivated in the greenhouse of Northwest A&F University, and sampled in September 2025. The male fertility of this line is regulated by temperature but unaffected by the photoperiod [72]. The plants were subjected to a low-temperature environment (17/13 °C) 1 week prior to meiosis, which resulted in sterile pollen, which was designated as YSS. Conversely, fertility was restored to the plants under a higher temperature regime (24/20 °C), and they were designated YSF. The anthers at the three developmental stages uninucleate (U), binucleate (B), and trinucleate (T) were collected for total RNA extraction. The total RNA was isolated using the RNAiso Reagent (TaKaRa, Beijing, China) with three biological replicates. The cDNA was synthesized using the Evo M-MLV RT Mix Kit with gDNA Clean for PCR (Accurate Biology, Changsha, China). Quantitative real-time PCR (qRT-PCR) was performed using the QuantStudio™ 7 Flex Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA), and the levels of relative gene expression were calculated using the $2^{-\Delta\Delta C_t}$ method with *TaACTIN* as the reference gene. The primers were designed Oligo 7, and their sequences are listed in Table S12.

4.8. Silencing of *TaYUCCA19* by BSMV-VIGS and the Phenotypic Analysis

Barley stripe mosaic virus-mediated gene silencing (BSMV-VIGS) was employed to silence *TaYUCCA19* in wheat anthers to validate its role in male fertility as previously described [73]. Four vectors, including α , β , γ , and γ -phytoene desaturase (PDS), which were retained in our laboratory, were used in this study. A 218 bp fragment of *TaYUCCA19* was cloned into the γ vector by homologous recombination. The restriction enzyme MluI was used to linearize the α and γ plasmids; SpeI was used to linearize the β plasmid, and BssHII was employed to linearize both the γ -*TaYUCCA19* and γ -PDS plasmids. The RiboMAX™ Large Scale RNA Production System-T7 (Promega, Madison, WI, USA) was used for the in vitro transcription of the linearized plasmids. The wheat line YS3038 was grown under fertile conditions until the flag leaf had fully emerged. In vitro transcribed RNAs from each of the three plasmids per combination (0.5 μ L each) were mixed with 9 μ L GK-P buffer (50 mM glycine, 1% bentonite, 30 mM K₂HPO₄, and 1% diatomaceous earth) to prepare the inoculation solution. Each inoculation solution was applied to one flag leaf, and 10 plants were treated per group. GK-P buffer alone served as the mock control; the α , β , and γ vector combination served as the negative control; the α , β , and γ -PDS combination served as the positive control, because PDS silencing disrupts carotenoid biosynthesis, resulting in white bleaching of leaves, which visually confirms effective gene silencing. The α , β , and γ -*TaYUCCA19* combination served as the treatment group. Phenotypic changes

in the leaves were observed 14 days post-inoculation, while other aspects of plant growth and morphology were not affected. The anthers were collected at the trinucleate stage and crushed. They were then stained with a solution I₂–KI to evaluate the fertility of pollen. Pollen grains that were uniformly stained dark black and exhibited a plump, near-spherical morphology were classified as normal pollen, whereas pollen grains that weakly stained as shown by their yellow–brown coloration and irregular or shriveled morphology were classified as abnormal pollen. Subsequently, a qRT-PCR analysis was performed using uninucleate stage anthers to determine the silencing efficiency of the target gene. Finally, the seed set rates of the negative control and treatment groups were analyzed statistically. The primer and sequence information are provided in the Table S12.

4.9. Statistical Analysis

Experiments were conducted with three independent biological replicates for most measurements, and data are presented as the mean $\pm$ SD. Statistical significance was assessed using Student's *t*-test for most measurements, and Fisher's exact test for pollen grain counts after gene silencing. ** indicates $p \leq 0.01$.

5. Conclusions

In this study, a total of 64 *TaYUCCAs* were identified through a genome-wide analysis and classified into three clades based on their phylogenetic relationships. An analysis of the duplication events indicated that segmental and tandem duplications together drive the expansion of the wheat *YUCCA* gene family, and all these genes have undergone purifying selection. The expression of the *TaYUCCAs* varied across different tissues and developmental stages of reproductive growth, which may be closely related to the diverse gene structures within each clade and the variation in *cis*-acting elements in their promoter regions. In the temperature-sensitive male sterile line YS3038, *TaYUCCA19*, *TaYUCCA22* and *TaYUCCA25* expression was significantly downregulated at the uninucleate stage of pollen development under sterile conditions, which was consistent with the results of transcriptome sequencing. Further gene-silencing experiments revealed that silencing of *TaYUCCA19* simultaneously led to reduced expression of *TaYUCCA19* and its two homologous genes, *TaYUCCA22* and *TaYUCCA25*, in anthers. The silenced plants exhibited abnormal pollen morphology, and I₂–KI staining indicated that the pollen grains were largely non-viable, leading to a significantly reduced seed-setting rate, ultimately resulting in complete male sterility. These results indicate that *TaYUCCA19* and its homologous genes play critical roles in wheat pollen development.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/plants15040664/s1>, Figure S1: Chromosomal distribution of *TaYUCCAs* in wheat; Figure S2: Distribution of *cis*-acting elements in the promoter regions of *TaYUCCAs*; Figure S3: Differential expression analysis of *TaYUCCA19*, *TaYUCCA22* and *TaYUCCA25* based on FPKM values; Figure S4: Phenotypes of leaves under different treatment combinations after BSMV-VIGS; Figure S5: Multiple sequence alignment of *TaYUCCA19*, *TaYUCCA22*, and *TaYUCCA25*. Table S1: Transcript IDs and gene names of *YUCCA* gene family members in multiple species; Table S2: Basic characteristics of *TaYUCCAs*; Table S3: Ka/Ks ratios and estimated divergence time for duplicated *TaYUCCA* gene pairs; Table S4: Ka/Ks ratios and estimated divergence time for orthologous *YUCCA* genes between wheat and *Aegilops tauschii*; Table S5: Ka/Ks ratios and estimated divergence time for orthologous *YUCCA* genes between wheat and *Triticum dicoccoides*; Table S6: Ka/Ks ratios and estimated divergence time for orthologous *YUCCA* genes between wheat and *Triticum urartu*; Table S7: Ka/Ks ratios and estimated divergence time for orthologous *YUCCA* genes between wheat and rice; Table S8: Predicted *cis*-acting regulatory elements in the promoters of *TaYUCCA* genes; Table S9: GO annotations of *TaYUCCAs*; Table S10: Relative expression levels of

TaYUCCAs in YS anthers at three developmental stages; Table S11: Effects of *TaYUCCA19* silencing on seed setting rate in wheat; Table S12: Primers used in this study.

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