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Molecular Mechanisms of the Rice HAM Domain Gene *OsHIPP16* in Ovule Development

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Abstract Transcriptomic analyses have identified many differentially expressed genes (DEGs) at different stages of ovule development, suggesting complex regulatory networks are involved. Studies have shown that genes related to cell component biogenesis, membrane-binding organelles, and reproductive regulation are highly expressed during ovule development, and the role of sporophyte tissue in female gametophyte development has been confirmed by gene expression profiles. Relevant studies have also discussed the interaction of *OsHIPP16* with other proteins and its potential regulatory functions in ovule development. Providing new insights into its molecular mechanisms, *OsHIPP16* plays a crucial role in rice ovule development through complex molecular and cellular events. The interaction of this gene with various regulatory networks and its expression patterns suggest that it contributes significantly to the formation of female gametophytes. These findings enhance researchers' understanding of the genetic and molecular basis of rice ovule development and provide potential applications for improving rice fertility and crop yield. The aim of this study was to elucidate the molecular mechanism of rice HAM domain gene *OsHIPP16* in ovule development and to understand its role in female gametophyte formation and function in rice (*Oryza sativa*).

Keywords *OsHIPP16*; Ovule development; Rice; Female gametophyte; Gene expression

1 Introduction

Ovule development in rice (*Oryza sativa*) is a critical process that significantly impacts the plant's reproductive success and seed yield. The ovule, which eventually develops into a seed post-fertilization, is essential for the continuation of the species and for agricultural productivity. Understanding the molecular events associated with ovule development can provide insights into improving rice fertility and yield. Recent studies have utilized RNA sequencing to profile gene expression during various stages of ovule development, identifying numerous differentially expressed genes (DEGs) that play crucial roles in this process. These genes are involved in cellular component biogenesis, membrane-bounded organelles, and reproductive regulation, highlighting the complexity of molecular and cellular events during ovule development (Wu et al., 2015). Additionally, the regulatory networks involving transcription factors, plant hormones, and epigenetic modifications have been shown to be critical in the formation and fertility of the female gametophyte (Yang et al., 2016).

HAM domain genes are a group of genes that encode proteins with a highly conserved domain known as the HAM (Histidine Acidic Motif) domain. These genes are involved in various developmental processes in plants, including ovule development. The HAM domain proteins are known to interact with other proteins and play roles in transcriptional regulation, signal transduction, and cellular differentiation. In rice, the expression of microRNAs (miRNAs) during ovule development has been shown to regulate the interaction between sporophytic tissue and the female gametophyte, indicating a complex regulatory network involving HAM domain genes (Wu et al., 2017). The identification and functional analysis of these genes can provide valuable insights into the molecular mechanisms underlying ovule development and fertility in rice.

By integrating data from various studies, this study aims to elucidate the role of *OsHIPP16* in the regulation of ovule development, identify key regulatory networks and pathways, and provide a comprehensive understanding

of the molecular events involved. This knowledge can contribute to the development of strategies for improving rice fertility and yield through genetic and biotechnological approaches.

2 The Role of HAM Domain Genes in Plant Development

2.1 General functions of HAM domain genes

HAM domain genes, part of the GRAS family of transcriptional regulators, play crucial roles in the development and maintenance of shoot apical meristems (SAMs) in land plants. These genes are essential for the initiation and proliferation of stem cells within the SAM, which are responsible for the continuous formation of aboveground plant organs. The HAM family members are involved in dictating shoot stem cell initiation and proliferation, and their expression domains are shaped by specific signaling cascades (Geng and Zhou, 2021a; Geng et al., 2021). The N-terminal regions of HAM proteins, although variable and divergent, are important for their conserved functions across different plant lineages (Geng and Zhou, 2021b).

2.2 HAM domain genes in other plant species

The HAM domain genes are conserved across various plant species, including angiosperms, bryophytes, lycophytes, ferns, and gymnosperms. This conservation suggests that HAM genes originated before the divergence of bryophytes. In angiosperms, HAM genes have duplicated into two distinct groups, Type I and Type II, with Type II being widely present. Interestingly, HAM genes from non-angiosperms can replace the function of Type II HAM genes in Arabidopsis, indicating their conserved role in maintaining SAMs and promoting new stem cell niches (Geng et al., 2021). In Brassica napus, a genome-wide survey revealed the presence of 87 GRAS genes, including HAM subfamily members, which are involved in root development and stress response (Guo et al., 2019). In litchi, the *GRAS* gene family, including HAM subfamily members, plays roles in seed development and is regulated by miR171-mediated degradation (Figure 1) (Chen et al., 2021).

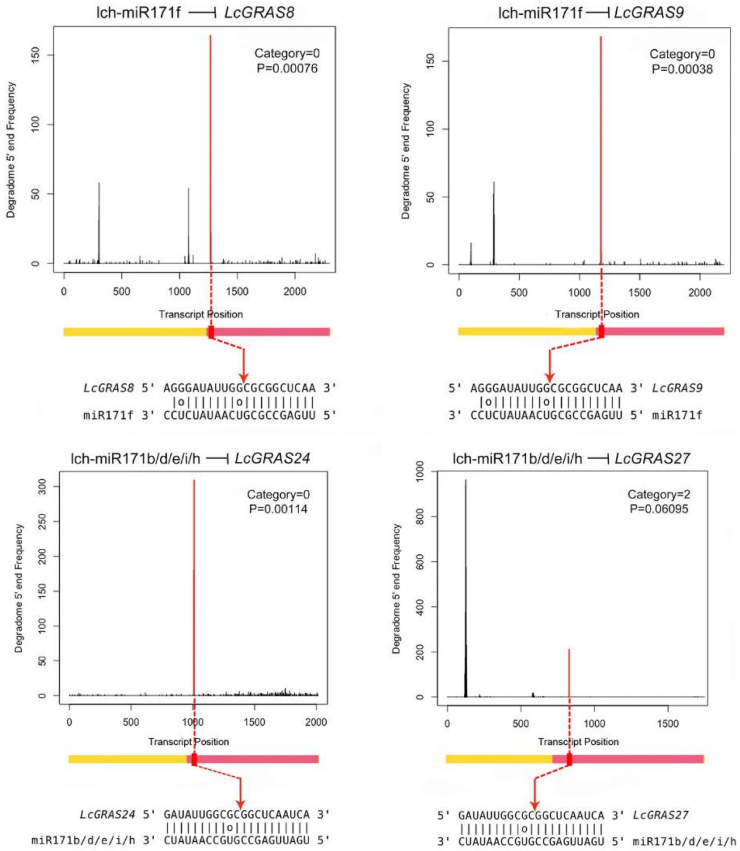

Figure 1 Target plots (t-plots) of identified miR171 targets in litchi using degradome sequencing (Adopted from Chen et al., 2021)

Image caption: T-plots from degradome data were shown in each panel, red lines indicate signatures consistent with miRNA-directed cleavage. The red vertical arrows point to the predicted cleavage sites. P: *P*-vaule. The yellow and pink color indicate the CDS region and the GRAS domain of the gene, respectively (Adopted from Chen et al., 2021)

2.3 Significance of HAM domain genes in reproductive development

HAM domain genes are significant in reproductive development, particularly in the regulation of floral organ induction and development. In sunflower, HAM genes are involved in the identity and formation of floral and inflorescence meristems, petals, stamens, and pistils (Shulga et al., 2008). In Arabidopsis, miR171a controls HAM1 functions within the protodermal cells of the embryo, which is essential for normal embryogenesis and proper organ formation (Takanashi et al., 2018). Additionally, HAM genes interact with WUSCHEL (WUS) family proteins to control stem cell functions in various meristems, including those involved in reproductive development (Zhou et al., 2014). The epigenetic modification of flowering-related genes by HAM1 and HAM2 further underscores their role in regulating flowering time and fertility in Arabidopsis (Xiao et al., 2013).

3 Functional Analysis of *OsHIPP16* in Rice

3.1 Discovery and characterization of *OsHIPP16*

The discovery of *OsHIPP16*, a member of the heavy metal-associated isoprenylated plant protein (HIPP) family, was part of a broader effort to identify genes involved in metal homeostasis and detoxification in rice. A comprehensive analysis of the rice genome revealed 54 HPP and HIPP genes, including *OsHIPP16*, which were differentially expressed under heavy metal stress conditions such as cadmium (Cd), manganese (Mn), and copper (Cu) (Khan et al., 2019). The functional characterization of *OsHIPP16*, along with other HIPP genes, was performed using yeast mutants sensitive to metal toxicity, demonstrating that these genes play a crucial role in metal accumulation and tolerance.

3.2 Expression patterns of *OsHIPP16* in rice

The expression patterns of *OsHIPP16* were studied under various metal stress conditions. Transcriptome analysis and quantitative real-time PCR (qRT-PCR) revealed that *OsHIPP16*, along with other HIPP genes, exhibited diverse expression patterns in response to excess Mn, Cu, and Cd stress (Khan et al., 2019). This differential expression suggests that *OsHIPP16* is actively involved in the plant's response to heavy metal stress, contributing to metal homeostasis and detoxification processes.

3.3 Genetic and molecular approaches used to study *OsHIPP16*

To elucidate the functional role of *OsHIPP16*, several genetic and molecular approaches were employed. These included the use of yeast mutants to test the gene's ability to confer metal tolerance, as well as the generation of rice mutants and transgenic lines with altered expression of *OsHIPP16*. The complementation tests in yeast mutants showed that cells expressing *OsHIPP16* accumulated more metals but exhibited improved growth under metal stress conditions. Additionally, the study of *OsHIPP16* mutants in rice under normal and metal stress conditions provided further insights into its role in metal detoxification and homeostasis (Khan et al., 2019). These approaches collectively highlight the importance of *OsHIPP16* in managing heavy metal stress in rice.

4 Molecular Mechanisms of *OsHIPP16* in Ovule Development

4.1 Regulatory pathways involving *OsHIPP16*

The regulatory pathways involving *OsHIPP16* in ovule development are complex and multifaceted. *OsHIPP16* is believed to interact with various transcription factors and signaling molecules that are crucial for the proper development of ovules. For instance, the AG subfamily gene *OsMADS13* has been shown to play a significant role in ovule identity determination in rice. Knock-out mutants of *OsMADS13* develop carpel-like structures instead of ovules, leading to female sterility, indicating that *OsMADS13* acts as a repressor of the carpel development pathway during ovule formation (Osnato et al., 2020). This suggests that *OsHIPP16* may be part of a broader regulatory network that includes *OsMADS13* and other related genes.

4.2 Interaction of *OsHIPP16* with other genes and proteins

OsHIPP16 likely interacts with a variety of genes and proteins to regulate ovule development. The interaction between *OsMADS13* and *OsMADS1*, for example, suggests a common set of target genes that are crucial for ovule development. Additionally, the involvement of Zinc-finger transcription factors, which are upregulated in the *OsMADS13* mutant, indicates that these factors could be potential partners or targets of *OsHIPP16* in the

regulatory network governing ovule development (Osnato et al., 2020). The integrated molecular network for ovule number regulation also highlights the role of various transcription factors, enzymes, and micro-RNAs, which could interact with *OsHIPP16* to influence ovule development (Qadir et al., 2021).

4.3 Phytohormonal influence on *OsHIPP16* activity

Phytohormones play a central role in the regulation of ovule development, and their influence on *OsHIPP16* activity is likely significant. Auxins (AUX), Brassinosteroids (BR), and Cytokinins (CK) are positive regulators of ovule number, while Gibberellins (GA) act negatively (Figure 2) (Qadir et al., 2021; Barro-Trastoy et al., 2022). These hormones could modulate the expression and activity of *OsHIPP16*, thereby affecting ovule development. The complex interplay between these phytohormones and *OsHIPP16* could be a key area of research to understand the precise molecular mechanisms underlying ovule development in rice.

5 Experimental Evidence Supporting *OsHIPP16* Function

5.1 Phenotypic analysis of *OsHIPP16* mutants

Phenotypic analysis of *OsHIPP16* mutants provides critical insights into the gene's role in ovule development. In rice, the D-lineage MADS-box gene *OsMADS13*, which is orthologous to *OsHIPP16*, has been shown to control ovule identity. Mutants of *OsMADS13* exhibit female sterility and transformation of ovules into carpelloid structures, indicating a loss of ovule identity (Dreni et al., 2007). This phenotypic evidence suggests that *OsHIPP16*, like *OsMADS13*, may play a crucial role in maintaining ovule identity and proper development.

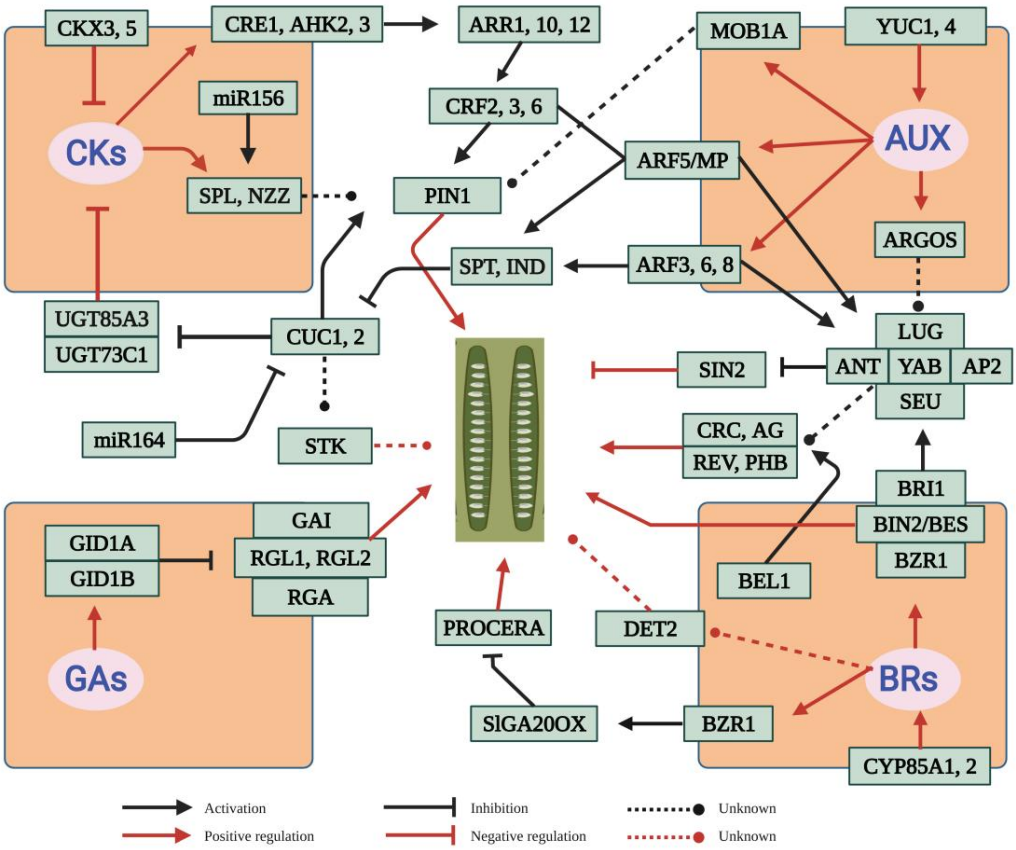

Figure 2 An integrated gene network for the regulation of ovule number (Adopted from Qadir et al., 2021)

Image caption: Four types of phytohormones (AUX, BRs, CKs and GAs) and other regulators are shown in blue and black color, respectively. The black and red arrows show the relationship between the up-/down-stream genes and between genes and phenotype, respectively. Functional characterization of "ovule number controlling genes" shows that a significant number play a role in biosynthesis and signaling pathways of several types of phytohormones, mainly as auxins (AUX), cytokinins (CK), brassinosteroids (BR), and gibberellins (GA) (Adopted from Qadir et al., 2021)

5.2 Overexpression and knockdown studies

Overexpression and knockdown studies are essential to understand the functional dynamics of *OsHIPP16*. In rice, the miRNA osa-miR171c targets four GRAS transcription factors, including *OsHAM* genes, to control the floral transition and maintenance of shoot apical meristem (SAM) indeterminacy (Fan et al., 2015). Overexpression of osa-miR171c leads to prolonged vegetative phases and delayed heading dates, while knockdown results in altered expression of key developmental regulators. These findings imply that manipulating *OsHIPP16* expression could similarly affect ovule development and floral transition, providing further evidence of its functional role.

5.3 Functional complementation assays

Functional complementation assays can validate the specific role of *OsHIPP16* in ovule development. In the case of *OsMADS13*, functional assays demonstrated that the gene's expression is restricted to ovules and that its mutation leads to significant phenotypic changes (Dreni et al., 2007). By conducting similar assays with *OsHIPP16*, researchers can determine whether the gene can rescue the ovule identity defects observed in mutants, thereby confirming its functional importance in ovule development.

6 Comparative Analysis with Other HAM Domain Genes

6.1 Similarities and differences in HAM domain gene functions

The HAM domain gene *OsHIPP16* in rice plays a crucial role in ovule development, similar to other HAM domain genes identified in various plant species. For instance, the D-lineage MADS-box gene *OsMADS13* in rice, which is orthologous to the Arabidopsis gene *STK* and Petunia genes *FBP7* and *FBP11*, is essential for ovule identity. Mutations in *OsMADS13* result in the transformation of ovules into carpelloid structures, indicating its critical function in maintaining ovule identity (Dreni et al., 2007). Similarly, the comprehensive transcriptome analysis of rice female-sterile line and wild-type line ovules has identified numerous differentially expressed genes (DEGs) that are involved in ovule development and fertile female gametophyte formation, highlighting the complex regulatory networks that govern these processes (Yang et al., 2016). Additionally, microRNAs (miRNAs) have been shown to play significant roles in the regulation of ovule development in rice, further emphasizing the multifaceted nature of gene regulation in these tissues (Wu et al., 2017).

6.2 Evolutionary conservation of HAM domain genes

The evolutionary conservation of HAM domain genes is evident from the functional similarities observed across different species. The rice gene *OsMADS13* shares a high degree of sequence similarity with its Arabidopsis and Petunia counterparts, *STK*, *FBP7*, and *FBP11*, respectively. This conservation extends to their functional roles, as all these genes are involved in maintaining ovule identity (Dreni et al., 2007). The presence of conserved miRNA-mediated regulatory mechanisms during ovule development in rice also suggests an evolutionary conservation of these regulatory pathways across plant species (Wu et al., 2017). The identification of numerous DEGs associated with key metabolic and signaling pathways in rice ovules further supports the idea that these genes and their regulatory networks have been conserved through evolution to ensure proper ovule development and fertility (Yang et al., 2016).

6.3 Insights gained from cross-species comparisons

Cross-species comparisons have provided valuable insights into the molecular mechanisms underlying ovule development. The functional analysis of *OsMADS13* in rice has revealed its role in ovule identity and floral meristem determinacy, similar to the roles of *STK* in Arabidopsis and *FBP7/FBP11* in Petunia (Dreni et al., 2007; Rodríguez-Cazorla et al., 2018; 2020). This cross-species functional similarity underscores the importance of these genes in reproductive development. Additionally, the transcriptome analysis of rice ovules has identified key regulatory genes and pathways that are likely conserved across species, providing a broader understanding of the genetic and molecular basis of ovule development (Yang et al., 2016). The study of miRNA expression profiles during rice ovule development has also highlighted the potential for miRNA-mediated regulation to be a conserved mechanism in plant reproductive development, offering new avenues for exploring the regulation of ovule development in other species (Wu et al., 2017; Babaei et al., 2022).

7 Potential Applications in Rice Breeding

7.1 Enhancing ovule development for improved yield

The enhancement of ovule development is a critical factor in improving rice yield. The *OsHIPP16* gene, which plays a significant role in ovule development, can be targeted to increase grain size and yield. For instance, the OsSPL16 gene, which is synonymous with the quantitative trait locus GW8, has been shown to promote cell division and grain filling, leading to increased grain width and yield in rice (Wang et al., 2012). By understanding and manipulating similar pathways in *OsHIPP16*, breeders can potentially enhance ovule development, thereby improving overall yield.

7.2 Genetic engineering approaches

Genetic engineering offers a powerful tool for manipulating the *OsHIPP16* gene to enhance rice breeding outcomes. The use of CRISPR/Cas9 technology to edit genes such as OsSPL16 has demonstrated significant increases in grain yield by modulating the expression of key enzymes and proteins involved in cell cycle regulation and metabolism (Usman et al., 2020; Park et al., 2022). Applying similar CRISPR/Cas9 techniques to *OsHIPP16* could lead to targeted mutations that enhance ovule development and improve yield without affecting other agronomic traits.

7.3 Breeding strategies incorporating *OsHIPP16*

Incorporating *OsHIPP16* into breeding strategies can be achieved through marker-assisted selection and other advanced breeding techniques. The success of marker-assisted strategies in targeting elite alleles of genes like GS3 and OsSPL16 to improve grain size and quality (Wang et al., 2012) suggests that similar approaches could be used for *OsHIPP16*. By selecting for beneficial alleles of *OsHIPP16*, breeders can develop rice varieties with enhanced ovule development and higher yields.

8 Future Directions and Research Gaps

8.1 Unresolved questions in *OsHIPP16* research

Despite significant advancements in understanding the role of *OsHIPP16* in rice development, several questions remain unanswered. One major gap is the precise molecular mechanism by which *OsHIPP16* influences ovule development. While it is known that hybrid proline-rich proteins (HyPRPs) like *OsHIPP16* are involved in stress responses and developmental processes, the specific pathways and interactions in ovule development are not fully elucidated (Kapoor et al., 2019). Additionally, the potential redundancy and interaction with other HyPRPs or related proteins in rice need further exploration. The role of *OsHIPP16* in the broader context of reproductive development, including its interaction with other key regulatory genes such as *OsMADS13* and OsGCD1, also remains to be clarified (Dreni et al., 2007; Huang et al., 2017).

8.2 Advanced techniques for studying *OsHIPP16*

To address these unresolved questions, several advanced techniques can be employed. CRISPR/Cas9 gene editing can be used to create precise knockouts or modifications of *OsHIPP16* to study its function in ovule development (Huang et al., 2017). RNA sequencing (RNA-seq) combined with genetic subtraction can provide insights into the differential expression of genes in *OsHIPP16* mutants compared to wild-type plants, helping to identify downstream targets and pathways (Yang et al., 2016). Additionally, yeast two-hybrid (Y2H) screening and co-immunoprecipitation (Co-IP) assays can be utilized to identify and validate protein-protein interactions involving *OsHIPP16*, shedding light on its molecular partners and functional networks (Kong et al., 2019). In situ hybridization and immuno-localization techniques can further elucidate the spatial and temporal expression patterns of *OsHIPP16* during ovule development (Miyoshi et al., 2002).

8.3 Long-term goals for ovule development research

The long-term goals for research on ovule development in rice include a comprehensive understanding of the genetic and molecular networks that regulate this critical process. This knowledge can be leveraged to improve rice fertility and yield through targeted breeding and genetic engineering. One goal is to develop rice varieties with enhanced ovule development and seed setting, which could contribute to higher crop productivity (Wang et

al., 2017). Another objective is to explore the evolutionary conservation and divergence of ovule development mechanisms across different plant species, providing broader insights into plant reproductive biology (Dreni et al., 2007). Ultimately, integrating the findings from *OsHIPP16* research with other key regulatory genes and pathways will pave the way for innovative strategies to enhance rice reproduction and stress resilience (Fan et al., 2015; Kapoor et al., 2019; He et al., 2022).

9 Concluding Remarks

The *OsHIPP16* gene plays a significant role in the regulation of ovule identity and development in rice. Studies have shown that *OsHIPP16*, along with other MADS-box genes such as *OsMADS13*, is crucial for maintaining ovule identity and preventing the transformation of ovules into carpelloid structures. The expression of *OsHIPP16* is tightly regulated during different stages of ovule development, indicating its importance in the precise control of reproductive organ formation. Furthermore, the interaction of *OsHIPP16* with other transcription factors and miRNAs, such as osa-miR171c, highlights the complex regulatory networks involved in ovule development.

The findings from this study have significant implications for rice biology and agriculture. Understanding the molecular mechanisms governing ovule development can lead to the development of rice varieties with improved fertility and seed production. For instance, manipulating the expression of *OsHIPP16* and related genes could enhance ovule identity and prevent sterility issues, thereby increasing yield. Additionally, insights into the regulatory networks involving *OsHIPP16* can inform breeding strategies aimed at developing rice varieties with better stress tolerance and reproductive success under adverse environmental conditions. The knowledge gained from studying *OsHIPP16* can also be applied to other crops, potentially leading to broader agricultural benefits.

Research on *OsHIPP16* is crucial for advancing our understanding of the genetic and molecular basis of ovule development in rice. The gene's role in maintaining ovule identity and its interaction with other regulatory elements underscore its importance in reproductive biology. Continued investigation into *OsHIPP16* and its associated pathways will not only enhance our knowledge of plant developmental processes but also contribute to the development of high-yielding and resilient rice varieties. The potential agricultural applications of this research highlight the importance of *OsHIPP16* in ensuring food security and sustainable crop production.

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Conflict of Interest Disclosure

The author affirms that this research was conducted without any commercial or financial relationships that could be construed as a potential conflict of interest.

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