OsMYB80 Regulates Anther Development and Pollen Fertility by Targeting Multiple Biological Pathways

Xiaoying Pan1,4, Wei Yan1,2,4, Zhenyi Chang1,2, Yingchao Xu3, Ming Luo3, Chunjue Xu2, Zhufeng Chen2,*, Jianxin Wu1,4 and Xiaoyan Tang 1,2,*

1Guangdong Provincial Key Laboratory of Biotechnology for Plant Development, School of Life Sciences, South China Normal University, Guangzhou 510631, China
2Shenzhen Institute of Molecular Crop Design, Shenzhen 518107, China
3Guangdong Provincial Key Laboratory of Applied Botany, Key Laboratory of South China Agricultural Plant Molecular Analysis and Genetic Improvement, South China Botanical Garden, Chinese Academy of Sciences, Guangzhou 510650, China
4These authors contributed equally to this work.

*Corresponding authors: Xiaoyan Tang, E-mail, txy@frontier-ag.com; Fax, +86 020 85211372; Zhufeng Chen; E-mail, czf@frontier-ag.com; Fax, +86 2085211372.

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Pollen development is critical to the reproductive success of flowering plants, but how it is regulated is not well understood. Here, we isolated two allelic male-sterile mutants of OsMYB80 and investigated how OsMYB80 regulates male fertility in rice. OsMYB80 was barely expressed in tissues other than anthers, where it initiated the expression during meiosis, reached the peak at the tetrad-releasing stage and then quickly declined afterward. The osmyb80 mutants exhibited premature tapetum cell death, lack of Ubisch bodies, no exine and microspore degeneration. To understand how OsMYB80 regulates anther development, RNA-seq analysis was conducted to identify genes differentially regulated by OsMYB80 in rice anthers. In addition, DNA affinity purification sequencing (DAP-seq) analysis was performed to identify DNA fragments interacting with OsMYB80 in vitro. Overlap of the genes identified by RNA-seq and DAP-seq revealed 188 genes that were differentially regulated by OsMYB80 and also carried an OsMYB80-interacting DNA element in the promoter. Ten of these promoter elements were randomly selected for gel shift assay and yeast one-hybrid assay, and all showed OsMYB80 binding. The 10 promoters also showed OsMYB80-dependent induction when co-expressed in rice protoplast. Functional annotation of the 188 genes suggested that OsMYB80 regulates male fertility by directly targeting multiple biological processes. The identification of these genes significantly enriched the gene networks governing anther development and provided much new information for the understanding of pollen development and male fertility.

Keywords: DAP-seq • Male sterility • Oryza sativa • OsMYB80 • Pollen development • RNA-seq.

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Introduction

Anther development involves a series of events from the formation of stamen primordium to the release of mature pollen during anther dehiscence (Zhang et al. 2011). During early anther development, stamen primordial cells divide and differentiate into pollen mother cells (PMCs) surrounded by four layers of anther wall cells, from surface to interior the epidermis, endothecium, middle layer and tapetum (Zhang et al. 2011). The PMC undergoes meiosis, forming four haploid microspores that are initially enclosed as a tetrad by callose wall (Ariizumi and Toriyama 2011). Subsequently, the callose wall is degraded by callase produced by the tapetum, and individual microspore is released into the anther locule (Wan et al. 2011). During further development, microspores go through cell enlargement, vacuolation and two rounds of mitosis and eventually form two small sperm cells enclosed by a large vegetative cell full of nutrients, while outside of the microspore is covered by a wall that is gradually thickened till the pollen grain matures (Zhang et al. 2011). In accompany with microspore development after meiosis, the tapetum goes through the programmed cell death (PCD) and actively synthesizes and secretes materials for pollen wall formation and pollen maturation (Shi et al. 2015a).

The mature pollen wall generally has two structural layers: the inner intine and the outer exine (Ariizumi and Toriyama 2011). The intine is believed to be formed by the male gametophyte and...
consists of cellulose, hemicellulose and pectic polymers similar to the primary walls of common plant cells (Ariizumi and Toriyama 2011). The exine commonly comprises two layers, the outer sexine and the inner nexine. The sexine contains an outermost roof, the tectum, and radially directed rods, the bacula, which together determine the species-specific appearance of pollen grains. The nexine consists of layers of nexine I, on which the bacula is anchored, and nexine II, which is laid down on the surface of pollen grains. It is mainly composed of long polyhydroxylated aliphatic chains and of small amounts of aromatic rings derived from the phenylpropanoid metabolism (Dominguez et al. 1999).

Recent genetic studies in Arabidopsis and rice have identified a large number of evolutionarily conserved genes that are required for pollen development and male fertility (Shi et al. 2015a). Among them, a group of genes is involved in the biosynthesis of aliphatic lipids and phenolic compounds. For example, Arabidopsis CER1 and its rice ortholog OsCER1 (Aarts et al. 1997, Ni et al. 2018), Arabidopsis CER3/WAX2/YRE/FLP1 and its rice ortholog WDA (Jung et al. 2006, Rowland et al. 2007) and Arabidopsis CER6/CUT1 (Fiebig et al. 2000) are involved in the biosynthesis of very-long-chain fatty acids and alkanes, which are precursors of aliphatic polymers. Arabidopsis AtGPAT1 and its rice homolog OsGPAT3 encode glycerol-3-phosphate acyltransferases that play key roles in regulating lipids and extracellular lipid polyesters by generating lysophosphatidic acids and acylating glycerol 3-phosphate at the sn-1 or sn-2 hydroxyl with acyl-CoA or acyl carrier protein (ACP) to alter glycerolipid triacylglycerol biosynthesis (Zheng et al. 2003, Ni et al. 2018), Arabidopsis AtUGP2 and their rice orthologs OsUGP1 and OsUgp2 encode UDP-glucose pyrophosphorylases that are essential for callose wall formation during meiosis (Chen et al. 2007, Park et al. 2010). Rice SPG2/IRX9L encodes a family GT43 glycosyltransferase involved in xylan backbone biosynthesis, while UPEX1 encodes a family GT31 glycosyltransferase likely involved in the galactosylation of arabinoxylan polymers; both SPG2 and UPEX1 are required for primexine synthesis (Li et al. 2017, Suzuki et al. 2017). Rice Osg1 encodes β-1,3-glucanase for the degradation of callose wall surrounding the microspore (Wan et al. 2011). QRT1 and QRT3 encode a pectin methylesterase (pectinase) and an endo-polygalacturonase, respectively, that act sequentially to degrade the cell wall surrounding PMCs, and mutation of either gene results in the formation of pollen grains that are not separated (Rhee et al. 2003, Francis et al. 2006). Other genes such as OsUAM3 encoding a UDP-arabinopyranose mutase (Sumiyoshi et al. 2015), GTL1 encoding a glycosyl transferase (Moon et al. 2013) and CAP1 encoding an arabinoxylanase-like protein (Ueda et al. 2013) act in the microsporecyte. Many genes with functions in transportation were also found to be required for pollen fertility. In the past few years, several plant ABCG proteins, such as AtABCG11 (Panikashvili et al. 2010), AtABCG26 (Quilichini et al. 2014), AtABCG9, AtABCG31 (Choi et al. 2014), AtABCG1 and AtABCG16 (Yadav et al. 2014) in Arabidopsis and OsABCG26 (Zhao et al. 2015, Chang et al. 2016b), OsABCG15 (Qin et al. 2013, Niu et al. 2013a) and OsABCG3 (Chang et al. 2018) in rice, have been shown to contribute to pollen wall development. In addition, RAFTIN1 associated with the Ubisch body, type III lipid transporters (LTPGs) and glycosyl phosphatidylinositol-anchored nonspecific LTPGs were also found to be required for pollen wall development (Wang et al. 2003, Kim et al. 2012, Huang et al. 2013). Additional transporters including sugar transporters (Hirose et al. 2010), UDP-GlcNac and UDP-GalNac transporter ROCK1 (Niemann et al. 2015) and magnesium transporters (Li et al. 2015) are probably involved in the transportation of nutrients for pollen development.

In addition to the catalytic enzymes and transport proteins, many genes required for pollen development are involved in signal transduction and transcriptional regulation, including hormone receptors, receptor-like kinases and transcriptional
regulator (Shi et al. 2015a). The Arabidopsis transcription factors DYSFUNCTIONAL TAPETUM1 (DYT1), DEFECTIVE IN TAPETAL DEVELOPMENT AND FUNCTION1 (TDF1), ABORTED MICROSPORES (AMS), AtMYB80 (also named AtMYB103 or AtMYB188) and MS1 form a transcriptional cascade regulating genes of sporopollenin synthesis (Zhu et al. 2011). DYTI is the most upstream regulator that directly regulates TDF1, TDF1 directly regulates AMS, AMS regulates AtMYB80 and AtMYB80 regulates MS1 (Zhu et al. 2011). Corresponding to these Arabidopsis transcription factors, rice UNDERDEVELOPED TAPETUM1 (UDT1/bHLH164) is orthologous to DYTI (Jung et al. 2005), TAPETUM DEGENERATION RETADATION (TDR/bHLH5) is orthologous to AMS (Li et al. 2006, Zhang et al. 2008a), OsMYB80 is orthologous to AtMYB80 (Phan et al. 2012) and PERSISTENT TAPETAL CELL1 (PTC1/OsMS1) is orthologous to MS1 (Li et al. 2011, Yang et al. 2019a). In addition to these proteins, other transcription factors have also been found essential for pollen development in rice, including the MYB family transcription factor GAMYB (Aya et al. 2009, Liu et al. 2010), TDR-INTERACTING PROTEIN2 (TIP2/bHLH142) (Fu et al. 2014, Ko et al. 2014), ETERNAL TAPETUM 1/Delayed TAPETUM DEGENERATION (EAT1/DTD/bHLH141) (Ji et al. 2013, Niu et al. 2013b), TDR-INTERACTING PROTEIN3 (TIP3) (Yang et al. 2019b) and TGA transcription factor OsTGA10 (Chen et al. 2018). GAMYB is involved in gibberellin-mediated regulation of pollen wall development (Aya et al. 2009). GAMYB and UDT1 work in parallel to positively activate the expression of TDR (Liu et al. 2010). TIP2/bHLH142 acts downstream of UDT1 but upstream of TDR and EAT1, and TIP2/bHLH142 can form a dimer with TDR to further activate EAT1 transcription by binding to its promoter (Fu et al. 2014, Ko et al. 2014). EAT1 and TIP3 also interact physically with TDR to affect the expression of genes related to tapetum PCD and pollen wall formation (Niu et al. 2013b, Yang et al. 2019b). OsTGA10 regulates tapetum development and pollen formation by interacting with TIP2 and TDR to affect the expression of AP25 and MTR (Chen et al. 2018). TDR not only modulates tapetum development and degeneration but also functions in aliphatic metabolism and pollen formation by directly activating the expression of its target genes OsCP1, OsC6 and OsCYP703A3 (Li et al. 2006, Zhang et al. 2008a, Zhang et al. 2010, Yang et al. 2014). PTC1/OsMS1 regulates tapetum development and pollen formation by affecting the expression of tapetum- and microspore-expressed genes (Li et al. 2011).

AtMYB80 is an R2R3 transcription factor that plays a critical role in anther development (Phan et al. 2012). AtMYB80 has been shown to bind the promoters of ACOS5, CYP703A2, CYP704B1, PKSA, PKSB, TKPR1 and MS2 that are involved in sporopollenin synthesis, the promoter of transcriptional regulator MS1, the promoter of A1 aspartic protease gene UNDEAD regulating tapetum PCD, and the promoters of two other genes (GLOX1 encoding a glyoxal oxidase and VANGUARD1 encoding a pectin methylesterase) of unknown functions in pollen development, either by itself or in association with AMS or other unknown transcription factors (Phan et al. 2011, Wang et al. 2018). OsMYB80 is homologous to AtMYB80 in sequence and can replace the function of AtMYB80 in regulating male fertility when expressed in Arabidopsis plant (Phan et al. 2012), but how OsMYB80 functions in rice has not been studied. In this study, we isolated two male-sterile mutants of the OsMYB80 gene, which enabled us to analyze the function of OsMYB80 in rice development. Here, we report that OsMYB80 regulates male fertility in rice by directly targeting a large number of genes in different biological pathways.

Results
Isolation of OsMYB80 mutants and analysis of OsMYB80 gene expression

We screened an ethyl methanesulfonate (EMS) mutant library derived from the indica rice Huanghuazhan (HHZ) and isolated two complete male-sterile mutants (HT5763 and H5524). Both mutants exhibited normal vegetative and floral development (Fig. 1A, B), except for the anthers being smaller without pollen (Fig. 1C–F). Both mutant plants pollinated with the wild-type (WT) pollen exhibited normal seed set, indicating normal female fertility. The F1 plants were completely fertile, and the F2 progeny exhibited a segregation ratio of 223:60 for HT5763 (χ²=0.0339) and 226:61 for H5524 (χ²=1.0344), suggesting that the male sterile phenotype was controlled by a single recessive gene for both mutants.

To identify the causal mutant genes, 30 sterile individuals in each F2 population mentioned above were selected for bulk sequencing (Supplementary Table S1). The sequenced data were analyzed using the Simultaneous Identification of Multiple Causal Mutations (SIMM) pipeline (Yan et al. 2017), which identified two different mutation sites in LOC_Os04g39470 (OsMYB80). The G to A mutation in HT5763 caused Glu74 (GAG) substitution by Lys (AAG), and the T to A mutation in H5524 caused Trp37 (TGG) substitution by Arg (AGG) (Fig. 1H). High-resolution melting (HRM) analysis of the F2 individuals indicated that both mutations co-segregated with the male sterile phenotype. For simplicity, further analyses were conducted with the HT5763 mutant.

To confirm the mutation, a complementation experiment was performed by introducing a 2,512-bp genomic DNA fragment, including 1,203-bp upstream region and 1,309-bp gene body of OsMYB80, into the HT5763 mutant plant. Five independent transgenic lines of homozygous mutation background showed normal anther and pollen fertility (Fig. 1G, H), indicating that the mutations in OsMYB80 were responsible for the male sterile phenotype.

Because osmyb80 mutants exhibited defects only in male fertility, to understand the devoted role of OsMYB80, we analyzed the tissue specificity and developmental expression patterns of OsMYB80 using quantitative reverse transcription PCR (qRT-PCR). OsMYB80 was barely expressed in root, stem, leaf, lemma, palea, sterile lemma and pistil tissues. In anthers, however, the gene showed a developmentally regulated expression pattern (Fig. 2). OsMYB80 mRNA was very low in anthers before meiosis (stage 7), quickly increased during meiosis (stage 8), reached the highest level when microspores were released from the tetrads (stage 9), then declined to a very low level
Fig. 1 Mutation of OsMYB80 induced no-pollen male sterility in rice. (A–F) Whole plants, panicles, anthers and pollen grains stained with I₂-KI were compared between the WT and osmyb80 mutant. (G, H) The anthers and pollen grains from osmyb80 mutant complemented with the OsMYB80 gene. Scale bars: 10 cm in (A); 2 cm in (B); 1 mm in (C), (E) and (G); 100 μm in (D), (F) and (H). (I) Identification of the causal gene in HT5763 and H5524 mutant plants. The top panel showed the distribution of Euclidean distance scores (ED⁶) of single nucleotide polymorphisms (SNPs) on chromosomes of HT5763 and H5524. The red lines represent the Loess regression curves of ED⁶ values. The colored dots indicate the mutations along the chromosomes. Arrow shows the position of OsMYB80 gene on chromosome 4. Gene structure and mutation sites in the OsMYB80 gene are shown. Black boxes represent exons, gray boxes represent untranslated regions (UTRs) and the lines between boxes represent introns. The DNA fragment for gene complementation is indicated by the line.
tapetum-produced materials for pollen exine formation and anther epidermis cuticle structure (Ariizumi and Toriyama 2011, Shi et al. 2015a). The deposition of sporopollenin precursors on the microspore surface and thickening of the pollen cell wall were clearly visible in the WT (Fig. 4E, G). However, in osmyb80, deposition of sporopollenin precursors on the primary cell wall of microspore was not detected (Fig. 4F) and pollen exine was not formed before the microspore degeneration (Fig. 4H). At the mature stage, both the WT and osmyb80 anther epidermis were covered by grid-like cuticle structures that looked like fingers under the transmission electron microscope. However, the finger-like structures were slightly shorter in osmyb80 than in WT (Fig. 4I, J). Consistently, the WT anther surface exhibited deeper gullies than the mutant anther in the grid-like structure under a scanning electron microscope (SEM) (Fig. 4K, L).

Transcriptome analyses of genes regulated by OsMYB80

As a transcription factor, OsMYB80 is expected to affect anther development by regulating the expression of downstream genes. To identify genes that might be regulated by OsMYB80, we performed two sets of comparative transcriptome analyses using RNA-Seq. One set was to compare the transcriptomes in anthers at stage 9 between the WT and osmyb80 mutant. The other set was to compare the WT anthers at stages 9 and 7, because OsMYB80 was highly expressed at stage 9 and barely detectable at stage 7 (Fig. 2). Each sample had three biological replicates; thus, nine transcriptome libraries were sequenced. A total of 45.2–63.22 million raw reads were generated for each library (Supplementary Table S2). After the removal of low-quality reads, the clean reads were aligned to the Nipponbare reference genome (MSU v7) and the numbers of reads covering each gene were calculated.

The expression patterns were highly consistent among the three replicates for each sample (Supplementary Fig. S1). The differentially expressed genes (DEGs) were extracted with fold change ≥2 and false discovery rate (FDR) <0.05. There were 226 upregulated genes and 2,313 downregulated genes in osmyb80 anther, compared with the WT anther at stage 9 (Fig. 5A and Supplementary Table S3). Comparison of DEGs in WT anthers at stages 7 and 9 resulted in 3,242 upregulated genes and 3,369 downregulated genes at stage 7 (Fig. 5B and Supplementary Table S4).

qRT-PCR assays were performed to validate the DEGs identified by RNA-seq, using RNA samples that were collected at a different time from those for RNA-seq. We randomly chose 20 DEGs, 12 genes from the osmyb80 vs. WT anthers at stage 9 and 8 genes from the WT anthers at stage 7 vs. stage 9. The qRT-PCR and RNA-seq data showed close agreement (Supplementary Table S5), indicating that the transcriptome data were highly reliable.

Because the mutation of OsMYB80 caused multiple phenotypical defects in anther development, these defects (e.g. premature tapetum PCD) might trigger abnormal expression of certain genes. On the other hand, DEGs between the WT anthers at stages 7 and 9 also included those that

Characterization of cellular defects in osmyb80 mutant anthers

To understand how OsMYB80 affects pollen development, we analyzed the semi-thin sections of HT5763 mutant anthers. No clear difference between the WT and osmyb80 was detected before stage 8a when microspore mother cells (MMCs) finished meiosis I and formed dyads (Fig. 3A–D). At stage 8b, MMCs underwent meiosis II and formed tetrads of haploid microspores in the WT (Fig. 3E). In contrast, microspores in the mutant tetrads were more condensed and exhibited an irregular shape (Fig. 3F). At the same stage, the tapetal layer was more condensed and stained darker in the mutant than in the WT (Fig. 3F), suggesting premature PCD. Microspores were released from the tetrads by stage 9 in the WT, but the mutant microspores degenerated, leaving debris in the locule (Fig. 3G, H). Subsequently, the WT microspores underwent vacuolation and two rounds of cell division, forming spherical pollen grains filled with cellular contents (Fig. 3I, K), but the mutant anther exhibited empty and shriveled locule lacking microspores (Fig. 3J, L).

To more precisely characterize the cellular differences, transmission electron microscopy (TEM) analysis was performed on anthers at stages 8b–12. The WT tapetum underwent PCD from stage 8a when highly condensed and almost completely degenerated by stage 11 (Zhang et al. 2011). Discrete organelles were clearly visible in the WT tapetal cells at stage 8b (Fig. 4A). However, the organelles in the mutant tapetal cells were apparently disorganized (Fig. 4B), indicating a more advanced PCD. The WT tapetum started to form Ubisch bodies at the inner surface at stage 9 (Fig. 4C) that gradually grew into electron-dense orbicules facing the locule (Fig. 4G). However, no Ubisch bodies were formed by the mutant tapetum and tapetal cell membrane was apparently incomplete at stage 10 (Fig. 4D, H). Ubisch bodies are proposed to export the

![Fig. 2 qRT-PCR analysis of OsMYB80 gene expression. Anthers were collected at developmental stages 6–12. Other tissues were harvested from plants at the flowering stage. OsACTIN1 was used as the internal control. Data are shown as mean ± SD (n = 3).](image-url)
were regulated by factors other than OsMYB80. Thus, to identify genes that were related to OsMYB80, we overlapped the DEGs between the two comparisons and extracted the shared genes with similar expression patterns, resulting in 1,644 upregulated and 65 downregulated genes associated with the OsMYB80 function (Fig. 5C, D and Supplementary Table S6). Importantly, the shared DEGs contained 22 known male sterility genes identified in rice (Supplementary Table S7).

AgriGO was used to identify enriched gene ontology (GO) terms of the OsMYB80-regulated genes. As shown in Fig. 5E, the DEGs were significantly enriched in transport functions, fatty acid and small-molecule metabolic processes, regulation of transcription, ubiquitin-dependent protein degradation process, oxidation and reduction and binding with proteins, lipids and coenzymes. Genes related to oxidoreductase activity, hydrolase activity specifically on glycosyl bonds and strictosidine synthase were also significantly enriched. The top 50 Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways associated with the DEGs are shown in Supplementary Fig. S2. The enrichment of these genes was consistent with the active synthesis and transportation of metabolites by the tapetum at stage 9 and the lack of exine formation in osmyb80 mutant anthers.

Fig. 3 Histological features of anther development in the WT and osmyb80 mutant. WT (A, C, E, G, I, K) and osmyb80 (B, D, F, H, J, L) anthers at developmental stages 7–12 are shown. Scale bar = 20 μm. DMs, degenerated microspores; Dy, dyad cell; E, epidermis; En, endothecium; M, middle layer; MP, mature pollen; Ms, microspores; T, tapetal layer; Tds, tetrads.
Identification of OsMYB80-binding sequences by DNA affinity purification sequencing

It is perceivable that the DEGs identified by RNA-seq include genes that are regulated directly as well as indirectly by OsMYB80. To identify genes that are directly regulated by OsMYB80, we used DNA affinity purification sequencing (DAP-seq) for genome-wide identification of OsMYB80-binding sites in vitro (Bartlett et al. 2017). The analysis identified 7,968 enriched 200-bp peaks with $-\log_{10}(P\text{-value}) \geq 5$ (Supplementary Table S8). In total, 3,968 (49.80%) of these peaks were located within 1.5 kb upstream of the annotated open reading frames (ORFs), 1,960 (24.60%) were located within 1 kb downstream of putative ORFs and the remaining (25.60%) were distributed in intergenic regions in the genome (Supplementary Fig. S3A). The enriched peaks were almost uniformly distributed in the upstream regions but decreased with the distance to the stop codon in the downstream regions (Supplementary Fig. S3B). There were at least 34 known rice male sterile genes associated with a DAP-fragment (Supplementary Table S9).

Overlap of the genes identified by DAP-seq with the OsMYB80-regulated DEGs revealed 296 shared genes, of which 287 were upregulated and 9 were downregulated by OsMYB80 (Supplementary Table S10). Because most MYB proteins were known to regulate gene expression by binding to the promoters (Prouse and Campbell 2012), we therefore focused on the OsMYB80-regulated DEGs with the DAP-seq fragments in the promoter region, which identified 188 genes in total (Supplementary Table S11). Fourteen genes carried two DAP-seq fragments and three genes carried three DAP-seq fragments in their promoters, suggesting multiple OsMYB80-binding sites in these gene promoters (Supplementary Table S11).

Identification of putative OsMYB80-binding motifs in the promoters

OsMYB80 is orthologous to AtMYB80 (Phan et al. 2012). Previously, Xu et al. (2014) identified two motifs in the AtMYB80 promoter (ATGTTT and TTTGTTA) that were required for AtMYB80 autoregulation of its promoter activity in anthers. Although OsMYB80 and AtMYB80 promoters showed low identity overall, two conserved DNA motifs were identified at similar positions in the OsMYB80 promoter that differ from the AtMYB80 motifs of only one SNP in each motif (Fig. 6A). To test if these two motifs were functional for OsMYB80 binding, we conducted an electrophoretic mobility shift assay (EMSA). As shown in Fig. 6B, both motifs showed strong interaction with OsMYB80. In addition to the two DNA motifs in AtMYB80 promoter and the two motifs in OsMYB80 promoter, AtMYB80 was shown to bind three other motifs, including MYB1AT (A/T AACCA), MYB1LPR (GTAGTT) and MYBPZM (MYBPZM-1/2, CC A/T ACC; MYBPZM-1,1, CCAACA) (Phan et al. 2011). In addition, a series of conserved R2R3 MYB-binding motifs were identified in previous studies, including MYBCORE (CTGTTG), MYBST1 (GGATA), MYBGAHV (TAACAA), MYBCOREATCYCB1 (AACCG), MYBHv1 (CAACCG), MRE (AACCTAA), MYBS (TATCCA), MBS1 (TA A/G CTC), MBS2 (CAACTG) and MBS3 (CCGTCG) (Lu et al. 2002, Phan et al. 2011). Hence, we analyzed if these cis-elements were presented in the DAP fragments on the promoters of DEGs in Supplementary Table S11. The search identified 154 DAP fragments in 140 gene promoters containing at least one putative MYB80- and/or R2R3 MYB-binding motif (Supplementary Table S12). The most abundant motif presented in the DAP fragments was MYBPZM (53), followed by MYB1AT (45), AtMYB80-1 (25), MBS1 (24) and MYBST1 (23) (Supplementary Fig. S4). The DAP fragments were also subjected to MEME assay, which revealed a number of other enriched DNA elements (Supplementary Fig. S5). The top four enriched motifs...
Fig. 5 Identification of OsMYB80-related DEGs. (A) Volcano plot of DEGs between the osmyb80 and WT anthers at stage 9 (M9 vs. W9). (B) Volcano plot of DEGs between WT anthers at stages 7 and 9 (W7 vs. W9). Red dots and blue dots represent significantly up- and downregulated genes, respectively. Black dots represent no-change genes. (C) Overlap of upregulated genes identified in (A) and (B). (D) Overlap of downregulated genes identified in (A) and (B). (E) Significantly enriched GO analysis of OsMYB80-related DEGs. The count of genes (left y-axis) and $-\log_{10}(FDR)$ (right y-axis) were shown for each enriched GO term.
(ACC T/A A/C C/T/G C/T/A ACC T/A C/A G/T/G T/A T/G T A/T GGT G/A/T and TAG G/C T A/G) were similar to the MRE motif or its complement (AACCTAA or TTAGGTT), and the fifth one (CCAAC T/C) is similar to the MYBPZM (CC A/T ACC and CCAACCA) motif. The other motifs had not been reported with a function (Supplementary Fig. 55).

Genes in Supplementary Table S12 were likely to be the candidate genes directly regulated by OsMYB80, because they showed OsMYB80-dependent differential expression in RNA-seq assay, their promoters bound OsMYB80 in DAP-seq assay and there were putative MYB80-binding motifs presented in their promoters. To further test their candidacy, we randomly selected 10 genes and tested the binding of their putative promoter elements with OsMYB80 using EMSA (Table 1). As expected, all of these promoter elements bound OsMYB80 (Fig. 7). All these motifs were then tested for OsMYB80 binding using the yeast one-hybrid assay, and all showed positive results (Fig. 8). We further tested if OsMYB80 could regulate the promoters harboring these motifs in rice protoplast. As shown in Fig. 9, all these promoters showed higher activities in the presence of OsMYB80 than in the absence of OsMYB80, further suggesting that OsMYB80 directly regulates these promoters.

**Discussion**

In this study, we isolated two osmyb80 mutant plants of male sterile phenotype. Histological analysis of the mutant anther sections showed premature tapetum PCD, no-pollen exine formation and microspore cell death at the end of the tetrad stage. These phenotypic defects were similar to what in the Arabidopsis atmyb80 mutant anther (Zhang et al. 2007). Our results showed that OsMYB80 was specifically expressed in rice anthers during the period of tetrad formation and microspore release from the tetrad, which was similar to the timing of AtMYB80 expression during the Arabidopsis anther development (Zhang et al. 2007). By comparing gene expression profiles, we found 1,644 upregulated and 65 downregulated genes associated with the OsMYB80 function. GO and KEGG analyses indicated that functions related to fatty acid/lipid/sporopollenin biosynthesis, transmembrane transport, protein degradation and transcriptional regulation were significantly enriched in the OsMYB80-regulated DEGs. These enriched gene functions were also similar to what had been reported in the atmyb80 mutant (Zhu et al. 2010, Phan et al. 2011). These similarities were consistent with the conserved function of OsMYB80 and AtMYB80 in regulating anther development.

Our DAP-seq assay identified 7,968 enriched genomic fragments associated with OsMYB80 protein in vitro. Strikingly, at least 34 known rice male sterile genes were associated with a DAP fragment (Supplementary Table S9). However, most of these male sterile genes did not show an OsMYB80-dependent differential gene expression in our RNA-seq analysis.

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**Table 1** List of experimentally validated OsMYB80-binding promoters

| Gene     | Description                  | Position | Motif                  |
|----------|------------------------------|----------|------------------------|
| LOC_Os04g39470 | OsMYB80               | −463/−457 | OsMYB80-1          |
| LOC_Os01g04080 | Expressed protein       | −427/−422 | OsMYB80-2          |
| LOC_Os04g51990 | Transferase family protein | −604/−600 | MYBCOREATCYCB1       |
| LOC_Os09g36730 | MYB family transcription factor | −820/−815 | MYB1AT              |
| LOC_Os01g27170 | Potassium transporter     | −322/−317 | MYBPZM              |
| LOC_Os08g38810 | BURF domain-containing protein | −705/−700 | MYBCORE, MYBCOREATCYCB1 |
| LOC_Os07g40830 | App1                       | −441/−436 | MYB1AT, MBS1         |
| LOC_Os08g20200 | Male sterility protein     | −506/−501 | MBS2, MYBPZM         |
| LOC_Os03g35920 | Expressed protein          | −163/−157 | MYB1LEPR, AtMYB80-1  |
| LOC_Os06g11135 | Gibberellin receptor GI2L | −147/−141 | MYBPZM              |
| LOC_Os01g50750 | Zinc finger, CSHC4-type domain-containing protein | −264/−239 | MYB1AT, MYBPZM       |

*Position represents distance of the validated motif to the start codon. The validated motifs are marked in bold.*
these male sterile genes are indeed regulated directly by OsMYB80 remains to be verified.

Overlap of genes identified by DAP-seq with the OsMYB80-regulated DEGs identified 188 DEGs carrying DAP-seq fragment(s) in the promoter region (Supplementary Table S11). Most of these DEGs contain one or a few putative DNA motifs in the DAP-seq fragments that were known to interact with MYB80 or other R2R3 MYB proteins. Some of these 188 OsMYB80-regulated genes were also identified by a gene co-expression network analysis using sporopollenin synthesis and transport genes as a guide, but none was identified by DAP-seq. One possibility is that these orthologous genes are not subjected to direct regulation by OsMYB80. Another possibility is that these genes were missed by the DAP-seq assay.

Our DAP-seq assay generated 7,968 enriched fragments. However, most of the genes associated with a DAP-seq fragment were excluded from our candidate gene list because they did not meet the differential gene expression criteria. Overlap of the genes identified by DAP-seq with the OsMYB80-regulated DEGs resulted in only 296 shared genes (Supplementary Table S10). It is possible that some of the 7,968 DAP-seq fragments were derived from in vitro artificial interaction of the DNA with OsMYB80 protein. It is also possible that some of the excluded genes identified by DAP-seq were indeed regulated by OsMYB80, but they were dismissed by our stringent differential gene expression criteria, which required ≥2-fold differential expression and FDR ≤0.05 in both sets of the RNA-seq comparisons. Furthermore, it was reported recently that AtMYB80 binds the promoters of CYP704B1, ACOS5 and TKPR1, but these genes did not show a differential gene expression in atmyb80 mutant (Wang et al. 2018). It was proposed that AtMYB80 needs to team up with other transcription factors to regulate the expression of these genes (Wang et al. 2018). This type of genes would also be identified by our DAP-seq assay but excluded by the RNA-seq assay.

Searching against published male sterility genes, we found only 3 of the 188 putative OsMYB80-regulated genes that had...
be reported with a function in pollen wall synthesis and pollen development in rice, including LOC_Os06g40550 (OsABCG15; Qin et al. 2013), LOC_Os08g20200 (RAFTIN1; Wang et al. 2003) and LOC_Os09g27620 (PTC1/OsMS1; Li et al. 2011, Yang et al. 2019a). Seven genes are orthologous to the Arabidopsis genes known to regulate pollen development. Two of them, LOC_Os09g10260 and LOC_Os04g39470 (AbA0), were orthologous to the Arabidopsis genes AtMYB7/4/32 (Preston et al. 2004); but none has been demonstrated with a function in pollen development in rice. Given the fact that pollen wall development is associated with the active synthesis of aliphatic lipids and phenolics and that OsMYB80 is required for pollen exine formation, many of these genes are expected to contribute to sporopollenin synthesis and exine formation in rice.

Seven candidate genes are involved in polysaccharide metabolisms (Supplementary Table S11), including glycosyl hydrolase, pectinesterase, xyloglucan endotransglucosylase, endoglucanase and β,1,3-glucanase. One of them (LOC_Os07g41650) is orthologous to the Arabidopsis QRT1, a pectinesterase required for the separation of pollen tetrads (Francis et al. 2006). As the osmyb80 mutant exhibited a defect in callose wall degradation, we speculate that LOC_Os07g43940 (encoding a β,1,3-glucanase) may play a role in degrading the callose wall surrounding the microspore. The other cell wall degradation enzymes may degrade the cell wall surrounding the tetrads or degrade the tapetum cell wall as a mean to recycle the tapetum cell wall components.

There are 27 candidate genes encoding proteins of various transportation functions (Supplementary Table S11). Two of them, LOC_Os04g09520 and RAFTIN1, were known to be essential for rice pollen wall development (Wang et al. 2003, Qin et al. 2013), while LOC_Os04g09520 is orthologous to Arabidopsis LTPG2 required for pollen wall development (Kim et al. 2012). Other genes, including three different lipid transfer proteins, four different proteins involved in vesicle transportation, several transporters for sugar, sucrose, amino acids, nucleotide, malate, metal-nicotianamine, K⁺, Na⁺ and other metal ions, aquaporin, H⁺/Na⁺ antiporter, plasma membrane ATPase and protein secretion protein SEC61, have not been reported with a role in pollen development. It will be interesting to test if mutations of these genes impact pollen development.

At least 25 candidate genes encode components of the ubiquitination and proteasomal degradation pathway (Supplementary Table S11). Two of them, LOC_Os08g28820 and LOC_Os09g10260 were orthologous to the Arabidopsis gene SKP1/ASK1 required for pollen development (Yang et al. 1999). The presence of so many components of the ubiquitination and proteasomal degradation pathway suggested active protein degradation during the OsMYB80-expression stage (stages 8–10), which may be associated with the active degradation enzymes (Francis et al. 2006). As the osmyb80 mutant exhibited a defect in callose wall degradation, we speculate that LOC_Os07g43940 (encoding a β,1,3-glucanase) may play a role in degrading the callose wall surrounding the microspore. The other cell wall degradation enzymes may degrade the cell wall surrounding the tetrads or degrade the tapetum cell wall as a mean to recycle the tapetum cell wall components.

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progression of PCD and exhaustion of the tapetum cells during this period (Zhang et al. 2011).

At least 28 genes encode proteins in signal transduction and transcriptional regulation (Supplementary Table S1). Only LOC_Os09g27620 (OsMS1/PTC1) has been reported with a role in pollen development in rice (Li et al. 2011, Yang et al. 2019a). LOC_Os09g36730 is orthologous to AtMYB4/7/32 that is required for pollen development in Arabidopsis (Preston et al. 2004). It is perceivable that some of these genes may directly regulate the DEGs identified by our RNA-seq assay. The presence of so many regulatory genes in this group explains why the number of OsMYB80-dependent DEGs was so much bigger than the number of candidate DEGs directly regulated by OsMYB80. Significantly, three genes (LOC_Os06g11135, LOC_Os07g06880 and LOC_Os09g28720) are homologous to GA receptor GID1 (Yoshida et al. 2018). It is known that GA plays a critical role in anther development and male fertility (Aya et al. 2009, Kwon and Paek 2016). Whether these GID1-like genes play a role in GA signaling during pollen development remains to be studied.

In addition to the above major functional groups, three genes encode putative pollen wall antigens (LOC_Os07g40830, LOC_Os07g40850 and LOC_Os07g40890), which are pollen cell wall proteins putatively involved in stress responses and metabolic processes during pollen development (Chen et al. 2016). Five genes, including LOC_Os03g57200 (glutathione S-transferase), LOC_Os07g46570 (glutaredoxin family protein), LOC_Os01g15490 (PAPS reductase), LOC_Os03g24600 (methionine sulfoxide reductase) and LOC_Os01g12800 (peroxisomal membrane protein), may be related to the redox homeostasis of the tapetal cells (Traverso et al. 2013). Three genes are probably related to cell death, including LOC_Os09g19710 (hypersensitive response-induced protein), LOC_Os02g07820 (senescence-associated protein) and LOC_Os02g33820 (abscisic stress-ripening protein ASR5). It is known that the production of reactive oxygen species is critical for tapetal PCD and pollen development in Arabidopsis (Smirnova et al. 2014, Xie et al. 2014). Miss regulation of the redox- and cell death-related genes may be associated with the abnormal PCD displayed by the osmyb80 mutant. Other than these genes, 2 proteases

Fig. 9 Activation of the 10 selected promoters by OsMYB80 in rice protoplast. (A) OsMYB80. (B-K) Ten randomly selected promoters. The selected gene promoters were constructed upstream of the LUC reporter gene and co-expressed with OsMYB80 in rice protoplast. The REN gene under 35S promoter was used as the internal control. The relative LUC/REN ratio was determined for each promoter. The ratio shown as mean ± SD (n = 4). CK indicates a basal signal without OsMYB80. The corresponding gene promoters are listed in Table 1.
(LOC_Os04g66590 and LOC_Os01g04710), 2 ribosome-inactivating proteins (LOC_Os02g37090 and LOC_Os03g47460), 2 RNA-binding proteins (LOC_Os02g37090 and LOC_Os03g47460), 3 proteins that act on lysine metabolism (LOC_Os02g23170, LOC_Os04g68380 and LOC_Os07g40620), 3 heat shock proteins (LOC_Os08g34340, LOC_Os03g56540 and LOC_Os07g33350), 14 genes of various other functions and 38 genes of unknown functions were also identified as OsMYB80 directly regulated genes.

In summary, OsMYB80 directly regulates a large number of genes that probably act on different aspects of pollen formation. Based on the phenotypic defects exhibited by osmyb80 mutant and the putative functions of the OsMYB80-regulated genes, we propose a schematic model to summarize the biological processes regulated by OsMYB80 (Supplemental Fig. S6). These processes include the biosynthesis of precursors for pollen exine; transportation of pollen exine precursors for pollen wall formation; transportation of small nutrient molecules to nurture the pollen cell growth; degradation of the cell wall surrounding PMCs and the tetrads for microspore separation; massive protein degradation, redox homeostasis and cell death gene expression associated with the tapetum PCd; and signal transduction and transcriptional regulation that regulate downstream events for pollen development. Most of the OsMYB80-regulated genes we identified here were not known to be involved in pollen development. The identification of these genes significantly enriched the gene networks governing anther development and provided much new insight into the molecular mechanisms governing anther development and male fertility.

Materials and Methods

Plant materials and growth conditions

The HHZ mutant library generated by EMS treatment (Chen et al. 2014) was used for the isolation of HTS763 and H5524 mutants. The mutants were cross-pollinated by the WT HHZ to produce the F1 plants, which further self-pollinated to generate the F2 populations. All plants were grown in the paddy field under normal conditions with regular care.

Characterization of the mutant phenotype

Plants and flowers at the mature stage were photographed using a Nikon D80 Digital Camera. For pollen fertility analysis, pollen grains at the mature stage were stained with F3-KI solution and photographed using a Nikon AZ100 microscope as described previously (Chang et al. 2016a). Female fertility was tested by manual cross-pollination of the mutant plants with WT HHZ pollen, using osnp1-1 male sterile mutant plants as control (Chang et al. 2016a). For transverse section and electron microscopic analyses of anthers, spikelets at different developmental stages were collected and treated as described by Chang et al. (2016b).

Identification of the causal genes

The F2 plants were grown to the mature stage, and 30 individuals of the male sterile phenotype were harvested for DNA isolation. An equal amount of DNA from each F2 plant was mixed for genome re-sequencing using the Illumina HiSeq 2000 platform. The re-sequencing data were processed using the SIMM pipeline (Yan et al. 2017).

Gene expression analysis

Rice tissues, including root, stem, leaf, lemma, palea, sterile lemma and pistil, were collected at the flowering stage. Anthers were harvested at different developmental stages as described by Chang et al. (2016b). Total RNA was extracted using TRizol reagent (Invitrogen, Waltham, MA, USA) and then reverse-transcribed using PrimeScript™ RT Reagent Kit with gDNA Eraser (Perfect Real Time) (Takara, Dalian, China) according to the manufacturer’s instructions. qRT-PCR analysis was conducted according to the previous descriptions (Pan et al. 2015). Primers for qRT-PCR are listed in Supplemental Table S13. The relative gene expression level was calculated using the comparative Ct method (Livak and Schmittgen 2001) with OsActin1 as the internal control. Three biological replicates were analyzed for each sample.

Transgenic complementation of osmyb80 mutant

The 2,512-bp OsMYB80 genomic DNA, including 1,203-bp upstream region and 1,309-bp gene body, was PCR-amplified using the OsMYB80-com primer pair (Supplemental Table S13) and HHZ genomic DNA as a template. The PCR product was cloned into the pMD19-T vector (Takara) and confirmed by sequencing. The plasmid was then digested with BamHI and HindIII, and the segment was then ligated into the binary vector PCMBA1300 predigested with the same enzymes. The construct was introduced into Agrobacterium tumefaciens AGL1 strain and transformed into rice calli derived from the off-spring of osmyb80 heterozygote plants. The positive transgenic lines were identified by PCR with primers Hyg-PCR (Supplemental Table S13) for the selection marker gene Hyg. To identify the background genotype of transgenic plants, primers com-background (Supplemental Table S13) flanking the 2,512-bp OsMYB80 DNA were used to amplify the genomic DNA and the PCR product was then diluted 1,000× for subsequent HRM analysis (Lochmann et al. 2011) using the primer pair OsMYB80-HRM (Supplemental Table S13).

Transcriptomic library construction and sequencing

Three replicates of osmyb80 mutant anthers at stage 9 (M9) and WT HHZ anthers at stage 7 (W7) and stage 9 (W9) were harvested from a number of plants, giving a total of nine samples (three tissues × three replicates). The samples were immediately frozen in liquid nitrogen prior to RNA extraction using TRIzol reagent. The RNA-seq data were processed as previously described (Sun et al. 2016). Genes with fold change ≥2 and the FDR of <0.05 were considered to be significantly differentially expressed. DEGs showing similar expression patterns between the two comparisons (M9 vs. W9 and W7 vs. W9) were extracted as candidate genes regulated by OsMYB80. The enriched GO terms of the overrepresented DEGs showing similar expression patterns were extracted as input (Moriya et al. 2007).

Bioinformatics analysis of transcriptomic data

The RNA-seq data were processed as previously described (Sun et al. 2016). Genes with fold change ≥2 and the FDR of <0.05 were considered to be significantly differentially expressed. DEGs showing similar expression patterns between the two comparisons (M9 vs. W9 and W7 vs. W9) were extracted as candidate genes regulated by OsMYB80. The enriched GO terms of the overrepresented DEGs showing similar expression patterns were extracted as input (Moriya et al. 2007). The DEGs showing similar expression patterns were extracted as candidate genes regulated by OsMYB80. The enriched GO terms of the overrepresented DEGs showing similar expression patterns were extracted as input (Moriya et al. 2007).

DAP-seq analysis

The DAP-seq analysis was performed according to the protocol described by Bartlett et al. (2017). Briefly, 5 μg HHZ genomic DNA was sheared into ~200-bp fragments and then ligated with the Illumina-based sequencing adaptors to form a DNA library. The library was examined for adaptor-ligation frequency by quantitative PCR (qPCR) before applied to DAP-seq assay. OsMYB80 ORF fused to the Halo affinity tag was expressed in vitro using Tn5 Tn5 SP6 Coupled Wheat Germ Extract System (Promega, Fitchburg, WI, USA). The HaloTag-OsMYB80 protein was then purified from nonspecific proteins in the expression system using the magnetic HaloTag ligand (chloroalkane) beads (Promega) and verified by Western blotting with the anti-HaloTag antibody (Promega). The purified protein was incubated with 500 ng adaptor-ligated genomic DNA library at 30°C for 2 h before washing away the unbound DNA fragments.
The samples were heated at 98°C for 10 min to release the OsMYB80-bound DNA, and the recovered DNA was then PCR-amplified with the indexed TruSeq primers (Illumina, San Diego, CA, USA). Indexed DNA samples were subsequently combined and size-selected to remove the residual adaptor dimers. Purified DNA libraries were then sequenced using the Illumina HiSeq sequencing platform.

**Identification OsMYB80-binding sequences and the associated genes**

To identify OsMYB80-binding sequences, the clean DAP-seq reads were aligned to the Nipponbare reference genome (MSU v7.0) with HISAT2 (Kim et al. 2015). The enriched peaks were identified using MACS2 (Zhang et al. 208Bb) with —log10(P-value) ≥ 5. Then, the regions of the peaks were defined based on the annotations of the reference genome. Those located within 1.5 kb upstream of a target element were synthesized as two antiparallel oligonucleotides with overhang sticky ends combining platform.

DNA libraries were then sequenced using the Illumina HiSeq sequencing platform.

**Electrophoretic mobility shift assay**

The recombinant protein His-OsMYB80 was purified from *Escherichia coli* BL21 using Ni-NTA Superflow columns (Qagen, Venlo, Netherlands) according to the manufacturer’s protocol. DNA probes were prepared by annealing a 5’-biotinylated oligonucleotide to a complementary unmodified oligonucleotide. The complementary oligonucleotides were diluted in annealing buffer (20 mM Tris–HCl, pH 8.0, 50 mM NaCl, 1 mM EDTA) to a final concentration of 20 μM, heated to 95°C for 5 min and cooled down at 25°C for 2–3 h. The same procedure was followed to generate unmodified dsDNA fragments for competition assays. Probes for selected genes are listed in **Supplementary Table S15**.

**The yeast one-hybrid assay**

The Matchmaker Gold Yeast One-Hybrid Library Screening System User Manual (Takara) was followed for the yeast one-hybrid assay. To construct the target-reporter plasmid (pBait-AbAi), tandem copies of the DNA target element were synthesized (Supplementary Table S15) and inserted upstream of the AbAi reporter gene in pBait-AbAi plasmid. The insert DNA was synthesized as two antiparallel oligonucleotides with overhang sticky ends compatible with the sticky ends of the pBait-AbAi vector predigested with HindIII and SalI. After construction, the pBait-AbAi plasmids were digested with SfiI and the linearized plasmids were transformed into YHGold yeast strain (Takara). The transformants were selected on a synthetic dropout-Ura plate and confirmed by colony PCR analysis using the Matchmaker Insert Check PCR Mix 1 (Takara) for fragment integration into the yeast genome. The resulting bait-reporter yeast strains were then examined for the minimal inhibitory concentration of Aureobasidin A (AbA).

To confirm the interaction between the DNA fragments and OsMYB80, the full-length coding sequence of OsMYB80 was cloned into the pGADT7 vector (Takara). The OsMYB80 construct or empty vector (negative control) was transformed into the YH bait-reporter strain and selected on a synthetic dropout-Leu plate. Activation of the reporter gene was measured as peaks in promoters, and those located within 1 kb downstream of a gene were defined as peaks in promoters, and those located within 1 kb downstream of a gene were defined as peaks in 3’-UTR. Peaks located with >1.5 kb from the boundaries of a gene were defined as peaks in intergenic regions.

**Protoplast transfection assay**

For the transient transcriptional activity assay, ~1,500-bp promoter sequences of the tested genes were PCR-amplified using primers in **Supplementary Table S16** and then constructed upstream of the LUC gene in pGreen II 0800-LUC vector using In-Fusion cloning method (Hellens et al. 2005). The renilla luciferase (REN) gene under the control of 35S promoter in the pGreen II 08000-LUC vector was used as the internal control (Yoo et al. 2007). The CDS sequence of OsMYB80 was PCR-amplified using the primer set OsMYB80-effector (**Supplementary Table S16**), digested with BamHI and XhoI and cloned into the pGreen II 62-SK vector (Nova Tech, Singapore) under the control of 35S promoter. Rice protoplast was prepared and transfected as previously described (Yoo et al. 2007). The firefly LUC and REN activities were measured using the Dual-Luciferase Reporter Assay System (Promega). The experiments were repeated at least four times, and each time with four independent measurements assessed.

**Supplementary Data**

Supplementary data are available at PCP online.

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**Disclosures**

The authors have no conflicts of interest to declare.

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