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Final report

project

Fertilisation-independent formation of embryo, endosperm and pericarp for apomictic hybrid rice

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1 Acknowledgments

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2 Executive summary

In July 1997, CSIRO and IRRI began a three-phase project to develop apomixis for hybrid rice (*Oryza sativa* L.), funded by ACIAR. The goal was to make the yield advantage and stress tolerance of hybrid rice available to poor farmers in Asia and Africa by using synthetic apomixis to reduce the cost and increase the flexibility of hybrid seed production. Phase 1 (July 1997-June 2002) involved three laboratories: CSIRO Horticulture in Adelaide, CSIRO Plant Industry in Black Mountain, and IRRI in the Philippines. It provided new insights into the similarities and differences between sexual reproduction and asexual apomixis. Highlights were (i) the identification of three *FERTILISATION-INDEPENDENT SEED (FIS)* genes in the sexual plant *Arabidopsis* that form part of a complex to repress endosperm formation in the absence of fertilisation and (ii) confirmation that FIS homologues exist in rice and in the natural apomict *Hieracium piloselloides* that reproduces by apospory (avoidance of meiosis).

Phase 2 (July 2003-June 2008) focused exclusively on rice and involved the Black Mountain Laboratory and IRRI. The three main objectives for Phase 2 were as follows:

Objective 1: To identify all of the known FIS orthologues in the rice genome and to functionally test each for efficacy in fertilisation-independent (FI) formation of the pericarp and seed coat of rice (Objective 1a) and FI formation of the embryo and the endosperm in the rice ovule (Objective 1b).

Objective 2: To develop FI embryos in the nucellus by inducing aposporous initials (AIs).

Objective 3: To combine the above traits to generate a basic form of apospory (meiotic avoidance) with embryo and endosperm induction in rice that could be refined further in Phase 3.

Both IRRI and CSIRO contributed to Objective 1a. They found that the checkpoint that normally prevents FI formation of the pericarp and seed coat could be bypassed using transgenic approaches. However, progress with these approaches was hampered by a background level of ovary enlargement in non-transgenic control plants. A change in rice variety from Nipponbare to one in which this background is negligible would be valuable.

Objective 1b was pursued by CSIRO. Based on sequence similarity with the *Arabidopsis FIS* gene, seven candidate rice FIS-like (*OsFIS*) genes were identified in the rice genome. Transgenic rice lines containing silencing constructs to down-regulate all of these *OsFIS* genes were generated and evaluated, and T-DNA insertion lines in three of the FIS-like rice genes were also evaluated. Unexpectedly, autonomous endosperm formation and autonomous embryo formation were not observed in any of the hundreds of transgenic rice lines analyzed. Data on expression of these genes, combined with an evolutionary analysis of the rice and *Arabidopsis* genes, indicated that rice FIS-like genes do not function in an equivalent manner to that found in *Arabidopsis* and its relatives. Repression of rice seed formation is likely to occur by an as yet unknown mechanism that needs further investigation if synthesis of apomixis is to be attempted (Luo et al, submitted).

Under Objective 2, the first milestone was to induce the formation of multiple secondary megaspore mother cells (MeMCs) in the nucellus. This milestone was reached (Zhao et al 2008) by silencing OsTDL1A, the rice orthologue of the *Arabidopsis* gene *TAPETUM DETERMINANT1*. IRRI showed that OsTDL1A-silenced lines produce secondary MeMCs in both indica and japonica genetic backgrounds. The second milestone is to convert secondary MeMCs into AIs by eliminating meiosis. IRRI has identified the rice orthologue of a gene required for initiation of meiosis in yeast. Silencing of this rice gene may prevent entry in meiosis and allow AI formation.

Because Objectives 1a, 1b, and 2 were not attained completely, Objective 3 has not yet begun and will be postponed until Phase 3, which should focus on integrating discoveries in rice with those in *Arabidopsis* and natural apomicts such as *Hieracium*.

3 Background

Origin of the project

The idea of using a synthetic form of apomixis to fix the yield advantage of hybrid rice originated with CSIRO (Peacock 1992). IRRI became interested in the idea after a workshop on apomixis was held at IRRI (Khush 1994). Both institutes had a long-standing interest in apomixis (Koltunow et al 1995, Brar et al 1995), and IRRI was playing a leading role in developing hybrid rice for tropical countries (Virmani 1994). The synthetic approach was attractive for rice because of the absence of apomixis in the germplasm of wild or cultivated rice (Brar et al 1995). ACIAR was approached to fund the project and a 15-year 3-phase project was developed and approved. ACIAR completed an ex ante impact assessment of the project during Phase 1 (McMeniman and Lubulwa 1997).

Outline of the three-phase project

In Phase 1 (July 1997-June 2002, with a no-cost extension to June 2003), the objective was to develop molecular tools to achieve synthetic apomixis in rice through studies on the model plant *Arabidopsis thaliana*, the model apomict *Hieracium piloselloides*, and rice itself. In Phase 2 (July 2003-June 2008), we would build on the accomplishments of Phase 1 to achieve apomictic hybrid seed production, based on fertilisation-independent embryogenesis in the nucellus of rice. In Phase 3 (July 2008-June 2113), the objective would be to integrate apomictic hybrid seed production into rice improvement programs at IRRI and then in Asian countries. Phase 1 of the project began in July 1997, and the mid-term review held in January 2000 favored completion of Phase 1 and continuation into Phase 2. The terminal review of Phase 1 confirmed continuation of the project, but Phase 2 was to focus exclusively on rice. Professor Peter Langridge (University of Adelaide) and Professor Hortz Lörz (University of Hamburg) were the external reviewers for Phase 1. Work on Phase 2 began in July 2003, with a mid-term review scheduled for 2006. Phase 2 terminated on 30 June 2008.

Importance of hybrid rice

The productivity of irrigated rice in farmers' fields has stagnated over the last 20 years. As a result, the rate of increase in rice production has fallen below the rate of increase in population, creating concern for future food security, particularly in Asia, where >90% of rice is grown and consumed and where rice accounts for >50% of total intake of calories. In China, hybrid rice gave a one-time increase of about 30% in rice production per hectare (Yuan 1998). Several other Asian countries are now attempting to reproduce this phenomenon (Tran and Nguyen 1998). Three-line hybrids and the newer two-line hybrids are grown on 16 million ha and 1 million ha, respectively, predominantly in China. The high cost and inflexibility of hybrid seed production are two of the major impediments to the wider spread of hybrid rice outside China. This situation is unfortunate, because the productivity of hybrid rice may increase considerably in the future as intrasubspecific hybrids (principally indica/indica) are superseded by intersubspecific and more distant hybrids that have a greater heterotic advantage (Khush et al 1998, Yuan 1998, Li and Yuan 1999).

Opportunity for research on "one-line" hybrid rice

Three-line hybrid seed production is costly and inflexible for rice because of the inefficiency of outcrossing in a naturally inbreeding crop and the need to use particular genetic resources for cytoplasmic male sterility and fertility restoration. Two-line hybrid seed production relies on environmental factors (photoperiod and/or temperature) to switch the female parent between male sterility and fertility. However, these cues are not very marked in the tropics (Lopez and Virmani 2000). Research to decrease the cost and

increase the flexibility and efficiency of these seed production methods is essential, but the above scenario justified the initiation of a project on one-line, or apomictic, hybrids. Because of the absence of apomixis in rice germplasm, some sort of synthetic approach was essential. It could be based on the discovery of genes controlling naturally occurring apomixis (Savidan 2000) or on an understanding of the genes controlling sexual reproduction in rice and *Arabidopsis*, or both. Because of the available expertise, CSIRO and IRRI decided to take both approaches, with *Hieracium* as the model apomict. Other groups around the world were studying apomictic grasses and were expected to publish data that would be helpful to us.

Rationale for Phase 2

Phase 2 builds on the achievements of Phase 1, including

(i) fertilisation-independent (FI) endosperm formation in *Arabidopsis* (CSIRO in 1998),

(ii) demonstration of interrelatedness of sexual and apomictic developmental pathways (CSIRO in 2002), and

(iii) FI enlargement of the pericarp and seed coat of rice (IRRI in 2001, CSIRO in 2002).

Because of these three achievements, it was clear at the time of submission of the Phase 2 proposal that two major objectives were to be attained before we could achieve synthetic apomixis for hybrid rice.

Objective 1—to achieve FI endosperm formation in rice using the RNAi approach to target endogenous *FIS* genes and then to combine this new trait with FI pericarp and seed coat formation to produce a full-size asexual seed with a large autonomous endosperm but as yet without an embryo.

Objective 2—to achieve FI embryogenesis.

During the first 3 years of Phase 2, Objectives 1 and 2 were to be pursued in parallel; then, during the last 2 years of Phase 2, we were to combine the outputs of Objectives 1 and 2 to achieve Objective 3, development of apomictic hybrid rice. Objective 4, which aims at effective communication and dissemination of research results, was to be pursued throughout Phase 2 using various approaches. After the Mid-Term Review, it was agreed that Objective 3 would have to be postponed until Phase 3.

4 Objectives

Original Objectives and Outputs for Phase 2

Objective 1: Fertilisation-independent (FI) formation of rice endosperm and pericarp

- Output 1.1 Refined FI pericarp formation based on understanding of OsAsp1 function
- Output 1.2 Refined FI endosperm formation based on RNAi of *OsFIS* genes
- Output 1.3 Lines combining FI pericarp formation and FI endosperm formation

Objective 2: FI embryogenesis in rice nucellus

- Output 2.1 Isolated rice orthologue of maize *Mac1* gene
- Output 2.2 Isolated rice orthologues of *Arabidopsis* CLV, WUS, STM, and AG genes
- Output 2.3 System to control genes of nucellus from megaspore mother cell (MMC1)
- Output 2.4 OsMac1-based system for inducing secondary MMC (MMC2)
- Output 2.5 WUS-induced FI embryogenesis in MMC2
- Output 2.6 WUS-induced FI embryogenesis in nucellar cluster proximal to MMC1

Objective 3: Apomictic hybrid rice

- Output 3.1 Line displaying FI formation of embryo, endosperm, and pericarp
- Output 3.1 Line in which endosperm feeds apomictic embryo during germination
- Output 3.3 One-line apomictic hybrid rice

Objective 4: Communication and dissemination of research results

- Output 4.1 Publications and Web site for pre-publication releases
- Output 4.2 Participation in Third International Symposium on Apomixis
- Output 4.3 Mid-term review at CSIRO
- Output 4.4 Participation in Fourth International Congress on Hybrid Rice
- Output 4.5 Terminal workshop at IRRI

Revised Objectives and Outputs agreed upon at Mid-Term Review

Objective 1: Convert secondary MMCs into aposporous initials for diploid embryo sac formation

- Output 1.1 Comparison of MSP1-RNAi and OsTPD1A-RNAi for stable induction of secondary MMCs in japonica and indica rice
- Output 1.2 Conversion of secondary MMCs into aposporous initials

Objective 2: Autonomous endosperm and embryo development

- Output 2.1 Characterization of *FIS*-RNAi transformants
- Output 2.2 Characterization of the T-DNA lines against OsCLF, OsEZA1 (OsiEZ1), Os-FIE1, OsVRN2 (OsEMF2a), and Os-MSI1.

5 Methodology

5.1 Location of research

The research was conducted in the Plant Breeding, Genetics, and Biotechnology Division of the International Rice Research Institute and in the Black Mountain Laboratory of CSIRO's Division of Plant Industry.

5.1.1 PBGB Division at IRRI

PBGB Division at IRRI is the leading public-sector entity for hybrid rice research outside China. Most of the germplasm being used for hybrid rice in South and Southeast Asia is derived from the hybrid rice program run for many years by Dr. Sant S. Virmani until his retirement in 2004. Since then, the hybrid rice group at IRRI has been led by Dr. Fangming Xie, who attended the mid-term review of this project in Canberra in August 2006. PBGB Division also has a long-standing interest in apomixis, with Dr. Darshan Brar having conducted the most thorough search for apomictic rice varieties among IRRI's large collection of diploid and polyploid accessions. PBGB Division is also known for its capacity to transform rice, including the indica varieties used in hybrid rice production.

5.1.2 Black Mountain Laboratory of CSIRO Plant Industry

CSIRO Plant Industry (PI) is Australia's largest plant science research organization. It carries out research at fundamental and applied levels to deliver improved plant-based food and fiber crops for agriculture and its track record of delivery is high. The concept of the synthesis of apomixis for rice arose from Dr. Jim Peacock, Plant Industry's chief, at a workshop held at IRRI in 1992. Apomixis became a core area of research at Plant Industry, and involved studies on the model sexual plant *Arabidopsis*, led by Dr. Abed Chaudhury, and later the apomict *Hieracium*, led by Dr. Anna Koltunow in Adelaide (when Plant Industry and CSIRO Horticulture merged in 1995). Dr. Chaudhury's lab was one of the first in the world to demonstrate that endosperm initiation could be uncoupled from fertilisation. Apomixis remains a strategic area of research in Plant Industry's Research Theme portfolio, and the work on apomixis gene isolation from natural apomicts is currently part of international collaborations involving India, Brazil, and New Zealand. Plant Industry carries out research on rice for the Australian rice industry, particularly on cold tolerance, and has international collaborations on rice research. Plant Industry has excellent transformation capability for rice research purposes and has participated in international programs to generate mutagenized rice populations and to characterize the function of rice genes for crop production.

5.2 Protocols employed

5.2.1 Bioinformatics

MSP1, EXS/EMS1, and TPD1 protein sequences were obtained from the NCBI database (www.ncbi.nlm.nih.gov) and used in tBLASTn searches (www.ncbi.nlm.nih.gov/blast/) to identify genes encoding closely related proteins in the *Arabidopsis* and japonica rice genomes. Deduced protein sequences were analyzed for subcellular location using tools available at http://us.expasy.org/tools/, together with SignalP-NN (Nielsen et al 1997) and PredSL (Petsalaki et al 2006). The deduced amino acid sequences were organized into multiple alignments and phylogenetic trees using CLUSTALW (http://clustalw.genome.jp) and TreeView (Page 1996).

Protein sequence comparison for Polycomb group proteins was performed using BLAST searches and multiple sequence alignments were performed with the Clustal W 1.8

program (Higgins and Sharp 1989). The phylogenetic trees were constructed using the MEGA 4 program (Tamura et al 2007).

5.2.2 Gene expression analysis by RT-PCR and GUS spatial expression analysis

Total RNA was extracted from different tissues by Trizol, according to the instructions from the manufacturer (Invitrogen, Carlsbad, CA). RNA was quantified as described by Ji et al (2005). RNA samples were then treated with RNase-free DNase (Promega, Madison, WI) to remove any contaminating genomic DNA. For gene-specific amplification of MSP1, OsTDL1A, and OsTDL1B by reverse transcription–polymerase chain reaction (RT-PCR), the following primer pairs were used (forward then reverse, written from 5′ to 3′): MSP1 (ATCTCCAGGTTTTTAGGCTTTACG, CTAGCAGGATGAAAAGCCAGAAAC); OsTDL1A (AACCCTACTACTACTCCTCC, TCATCACGTCCACCGTGTAC); and OsTDL1B (AGCTTGAGCAAGTATTTGGC, GAAGCCGATACGCTGGAACT). The locations of MSL1 primers within the genomic sequence are given in Supplementary Material, Figure S1A. In each case, the primers flanked introns to permit a clear distinction between expected RT-PCR and PCR products and to identify incomplete splicing. RT-PCR for each gene was performed with SuperScript™ One-Step RT-PCR with Platinum® Taq (Invitrogen), according to the manufacturer's instructions. The cytosolic glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as a control for successful amplification and absence of genomic DNA. Primers for the GAPDH gene were from Kathiresan et al (2002). RT-PCR products were separated by electrophoresis in 1.5% agarose gels, poststained using ethidium bromide, and viewed using Gel Doc™ XR System (Bio-Rad, Sydney, Australia).

5.2.3 Gene expression analysis by RNA in situ hybridization

Templates for RNA probe synthesis were prepared by cloning RT-PCR amplicons into the pGEM®-T Easy vector (Promega). The forward and reverse RT-PCR primers are given below and their locations in the genes are illustrated in Figure S1B: MSP1 (CTCGTAGCTATAGAGTAACCG, CACTTAGAAACAGGCAAGCAG), OsTDL1A (TCGAGTACACCAACTCCTTC, CTGTGAGTGATACTGACATG), and OsTDL1B (TCGGCTTCAACGACTGTCTG, GTACGTAGCTAATAGGCCAG). The clones were sequenced (Macrogen, Seoul, Korea) to determine fidelity and orientation. RNA probes (sense and antisense) were transcribed from the above plasmids using T7 and SP6 RNA polymerases (Promega). Transcription used 11-digoxigenin-UTP instead of UTP. RNA *in situ* hybridization was conducted on spikelets of approximately 3 mm, which were fixed in FAA solution, dehydrated, and sectioned as above. Sections were prehybridized and hybridized as described by Kathiresan et al (2002) and Ji et al (2005). After hybridization, slides were immersed in stop buffer (2X SSPE), cover slips were removed, and slides were washed and exposed for 2 h to anti-digoxigenin antibody that was conjugated to alkaline phosphatase (anti-dig-AP) (Roche Molecular Biochemicals, Indianapolis, IN). Sections were washed twice, spread with AP substrate solution, transferred into a dark humid box, and incubated overnight. Enzyme-substrate color reaction was terminated with TE buffer. Sections were dehydrated through a graded ethanol series and mounted. Blue hybridization signals on the tissues were viewed under a bright field in a microscope (Zeiss) supported by Image-Pro Plus software.

5.2.4 Microscopy protocols, including sectioning and histologcial staining

Spikelets at different stages were fixed overnight in FAA solution (10% [v/v] formaldehyde, 50% [v/v] absolute ethanol, 5% [v/v] acetic acid), dehydrated through a graded ethanol series, and embedded using paraffin (Paraplast Plus, Sigma Chemical Company, St. Louis, MO). Serial sections of 5-μm thickness were placed on Fisherbrand® Superfrost®/Plus microscope slides (Fisher Scientific, Hampton, NH) and incubated at 45 °C for 24 h. Sections were de-waxed in xylene, rehydrated through a graded ethanol series, and stained in fast green and safranin or aniline blue. Sections were viewed under

a bright field microscope (Carl Zeiss MicroImaging GmbH, Jena, Germany), supported by Image-Pro Plus 5.1 software (Media Cybernetics, Singapore).

5.2.5 Protein-protein interaction studies

5.2.5.1. Construction of plasmids

cDNA molecules corresponding to the longest putative open reading frames (ORF) of OsTDL1A and OsTDL1B were obtained by RT-PCR of RNA extracted from 3-mm spikelets. The following primer pairs were used (forward, reverse) and OsTDL1A (CCCTACTACTACTCCTCCTC, TTTCCTTGCGGCGATTGACG) and OsTDL1B (AGCTTGAGCAAGTATTTGGC, GAAGCCGATACGCTGGAACT). The amplicons were cloned into pGEM®-T Easy vector and sequenced. To create OsTDL1A, OsTDL1B, and MSP1 Gateway Entry clones (Invitrogen), PCR was conducted using gene-specific primers with 5′-extensions corresponding to sequences flanking the attB sequence. The PCR templates were the cDNA clones of OsTDL1A and OsTDL1B and a full-length cDNA clone of MSP1 (accession number AB103395, which was kindly provided by Dr. A. Miyao, National Institute of Agrobiological Sciences, Tsukuba, Japan). However, rather than expressing the entire MSP1 ORF, we created a truncated form of the protein (MSP1Δ), which consists of the first 894 amino acids of MSP1, that is, the N-terminus and the 34 LRR units but without the transmembrane and protein kinase domains. The attB-flanked PCR products were inserted into the pDONR™ 201 vector in the presence of BP clonase (Invitrogen). The inserts of the Entry plasmids were sequenced bidirectionally using primers targeting the pDONR vector in order to verify the sequence and the achievement of the desired reading frame. The following primers were used:

OsTDL1A attB1:

GGGGACAAGTTTGTACAAAAAAGCAGGCTTGAGGGTCTCCTCGGCGTCCAG.

OsTDL1A attB2:

GGGGACCACTTTGTACAAGAAAGCTGGGTTCTATGGGGCGGCGGGGTCGACG.

OsTDL1B attB1:

GGGGACAAGTTTGTACAAAAAAGCAGGCTTGGCCGACTGCACTACGATGCGTT.

OsTDL1B attB2:

GGGGACCACTTTGTACAAGAAAGCTGGGTTCTACTCACACGCGACATTAGCT.

MSP1 LRR attB1:

GGGGACAAGTTTGTACAAAAAAGCAGGCTTGGTATCCAATAGTTTCTGGCTTTTCA.

MSP1 LRR attB2:

GGGGACCACTTTGTACAAGAAAGCTGGGTATCCTGCAGCACAATCTGCCAAG.

5.2.5.2 Yeast two-hybrid assays

The method of James et al (1996), as modified by Luo et al (2000), was followed. Modified pACT2 and pAS2 yeast expression vectors that contain attR sites were used to generate the hybrid containing the GAL4 AD (a.a. 768–881) and GAL4 DNA-BD (a.a. 1–147), respectively. Target sequences of OsTDL1A and OsTDL1B were recombined into the pACT2 vector and MSP1 LRR region into the pAS2 vector by the LR reaction. Then, pACT2TDL1A and pACT2TDL1B were transformed into PJ69-4A yeast host stain using the lithium acetate procedure (James et al 1996). Successful introduction was verified by growing yeast on synthetic Drop-out medium plates lacking L-leucine. Using the same method, pAS2MSP1 and pACT2TDL1A or pACT2TDL1B were co-transformed into the yeast cells and selected on a double selective medium without L-leucine and L-histidine HCl. Strains showing protein-protein interactions were selected based on activation of the

HIS3 reporter gene on plates lacking leucine, tryptophan, and histidine in two independent experiments.

5.2.5.3 Bimolecular fluorescence complementation in onion cells

The method of Bracha-Drori et al (2004) was used. The Entry clones pDONR™ 201 TDL1A, pDONR™ 201TDL1B, and pDONR™ 201 MSP1 (see above) were also separately recombined into pBiFC GW cC1 and pBiFC GW nC1 vectors in the presence of LR Clonase (Invitrogen), respectively. BiFC vectors and the method used for bombardment of the gold particles coated by recombined plasmids into onion were all kindly provided by S. Curtin (CSIRO).

5.2.6 Transformation of japonica variety Nipponbare and confirmation of down-regulation of gene expression by RNA interference

To generate an RNA interference construct for the *OsTDL1A* gene, gene-specific primers were designed in the 3′ untranslated region. The primers produced a 409-bp amplicon through PCR and the use of the Platinum® Pfx DNA polymerase with proof-reading function (Invitrogen). The forward primer contained CACC at the 5′ end for TOPO cloning. Primers used for *OsTDL1A* were as follows (5′-to-3′, forward, reverse):

CACCGTACACGGTGGACGTGATGA, GAGTGATACTGACATGGGGT. The amplicon was cloned into the Gateway pENTR/D-TOPO cloning vector (Invitrogen), as described by Miki and Shimamoto (2004) and Miki et al (2005). The insert was sequenced for verification and transferred into the pANDA Destination vector by LR recombinase reaction. To allow stem-loop formation in the transcript from the RNAi cassette, the amplicon was inserted into the vector in opposite orientations at attB1 and attB2 recombination sites, flanking a partial GUS linker and marker sequence. After the recombinase reaction, the construct was transformed into *Escherichia coli* DH5α cells and recovered from kanamycin-resistant colonies. The correctness of cassette construction was verified by double digestion using SacI and KpnI, which cut unique restriction enzyme sites in the pANDA vector. Transgenic rice (*Oryza sativa* L. cv. Nipponbare) plants were produced by *Agrobacterium tumefaciens*-mediated transformation of rice primary embryogenic calli (Toki 1997). Transformants were selected and regenerated on media containing hygromycin B. Regenerated transgenic rice plants were grown in IRRI's Confinement-Level CL4 Transgenic Greenhouse under natural lighting conditions. Transformation was confirmed by PCR of the hygromycin phosphotransformation gene and the GUS marker in leaf samples taken at the vegetative stage of growth. Transcriptional activity of the cassette was established by RT-PCR of the partial GUS sequence; its effectiveness in down-regulating OsTDL1A transcript levels and its impact on OsTDL1B and MSP1 transcript levels were assessed by RT-PCR, using RNA extracted from 3-mm spikelets of T0 and T1 plants.

5.2.7 Endosperm imprinting analyses in rice

Nipponbare was emasculated and pollinated with IR64. Five days after pollination, we harvested the hybrid endosperm by cutting a small hole in the top side of 20 seeds and squeezing milky liquid into a grinding mortar. RNAs were isolated and RT-PCR products from the endosperm RNAs were subjected to direct sequencing using the corresponding primers with an Applied Biosystems Model 370A DNA Sequencer with fluorescent dye-labeled dideoxy terminators. PCR products amplifed from the hybrid endosperm cDNA of Nipponbare pollinated with IR64 were subcloned with pGEM T easy vector (Promega). Plasmid DNA from individual clones carrying the PCR fragment was sequenced.

5.2.8 Confirmation and analysis of rice DNA insertion lines

A gene-specific primer (OsCLF R) and right border primer (RB2) were used for PCR to confirm the T-DNA insertion in OsCLF (PFG_3A-60654, Postech). A pair of gene-specific

primers (OsCLF F and OsCLF R) flanking the T-DNA insertion were used to screen for homozygote mutants. The primer sequences are RB2: GGACCTGCATATAACCTGCA; OsCLF F: GCCTTCCGCCCTCCT; and OsCLF R: CGGTCCGATGTGATTTTCTT. A gene-specific primer (OsEMF2b ft R) and left border primer (LB French) were used for PCR to confirm the T-DNA insertion in OsEMF2b (DAE7C07, Genoplante). A pair of gene-specific primers (OsEMF2b ft F and OsEMF2b ft R) flanking the T-DNA insertion were used to screen for homozygote mutants. The primer sequences are LB French: CGCTCATGTGTTGAGCATAT; OsEMF2b ft F: TCTTTTGGGGCAGAAGTCAT; and OsEMF2b ft R: CACACGCTAATGGTCTGCTC . A gene-specific primer (OsFIE1 ft R) and left border primer (LB French) were used for PCR to confirm the T-DNA insertion in OsFIE1 (DAL3E12, Genoplante). A pair of gene-specific primers (OsFIE1 ft R and OsFIE1 ft R) flanking the T-DNA insertion were used to screen for homozygote mutants. The primer sequences are OsFIE1 ft F: TAAATGGCTTGGGGACTTTG, and OsFIE1 ft R: GGATTGGAAAACTTGCACTAGC.

5.3 Staff involved in the research

5.3.1 At IRRI

Dr. John Bennett (project leader, senior scientist)

Dr. Philippe Hervé (biotechnologist)

Ms. (later Dr.) Xinai Zhao (Ph.D. student, later postdoctoral fellow)

Mr. We Zhou (Ph.D. student)

Mr. Rico Gamuyao (M.Sc. student)

Ms. Justina de Palma (researcher)

Ms. Gina Borja (technician)

Ms. Rowena Oane (researcher)

Mr. Benni Malabanan (technician)

5.3.2 At CSIRO

Dr. Abdul Chaudhury (project leader, principal research scientist)

Dr. Ming Luo (postdoctoral scientist)

Ms. Yi Sao (technician)

Ms. Min Huang (technician)

6 Achievements against activities and outputs/milestones

Objective 1: Fertilisation-independent (FI) formation of rice endosperm and pericarp

no.	activity	outputs/ milestones	completion date	comments
1.1 PC	Refined FI pericarp formation based on understanding of OsAsp1 function	Bi X, Khush GS, Bennett J. 2005. The rice nucellin gene ortholog OsAsp1 encodes an active aspartic protease without a plant-specific insert and is strongly expressed in early embryo. Plant Cell Physiol. 46:87-98.	Published January 2005	IRRI found that transformation of rice with a construct containing a fusion between the *GUS* reporter gene and the promoter plus first exon and intron of *OsAsp1* gene caused fertilisation-independent (FI) enlargement of the pericarp and seed coat. However, non-transformed controls also showed this "pseudograin" trait, but at a lower frequency and with less reproducibly. In addition, the trait was accompanied by sterility, presumably because of silencing of OsAsp1, which is strongly expressed in very early embryos (Bi et al 2005). For these reasons, and because CSIRO had discovered a way of producing "pseudograins" without sterility using *OsFIS* genes, IRRI's research in this area was abandoned. However, CSIRO also encountered "pseudograins" occasionally in the non-transformed controls of the same genetic background (Nipponbare). This approach may give clearer results in another rice variety selected for the absence of "pseudograins" in control plants. A similar situation was observed in *Arabidopsis* by CSIRO.
1.2 A	Refined FI endosperm formation based on RNAi of *OsFIS* genes		A manuscript has been submitted (Luo et al)	Based on sequence similarity, all the FIS-like genes in rice had been identified, and transgenic lines with silencing constructs for each *OsFIS* gene had been evaluated. T-DNA insertion lines in three of the FIS-like genes in rice had also been evaluated. However, no autonomous endosperm formation had been obtained from all the transgenetic lines and the T-DNA insertion lines. The analysis of gene expression, imprinting in endosperm, and the evolution of all FIS homologs in rice indicate that FIS repression is a unique mechanism acquired in evolution in *Arabidopsis* and its relative species. There are no true *FIS* genes in the rice genome. Thus, a rice central cell may be repressed by another yet unknown mechanism.
1.3 PC, A	Line combining FI pericarp formation and FI endosperm formation			As reported in 1.2, this approach was abandoned because of sterility (IRRI) and "pseudograins" in control plants (IRRI, CSIRO).

PC = partner country, A = Australia

Objective 2: FI embryogenesis in rice nucellus

no.	activity	outputs/ milestones	completion date	comments
2.1 PC	Isolated rice orthologue of maize *MAC1* gene	Zhao XA, de Palma J, Oane R, Gamuyao R, Luo M, Chaudhury A, Hervé P, Xue QZ, Bennett J. 2008. OsTDL1A binds to the LRR domain of rice receptor kinase MSP1 and is required to limit sporocyte numbers. Plant J. 54:375-387	Published May 2008 (Zhao et al 2008)	MSP1 isolated by Nonomura et al (2003) appears not to be OsMAC1 (Ma et al 2007). It is therefore likely that OsMAC1 is a protein that interacts with MSP1 and, when mutated, produces the same phenotype as the msp1 mutant. OsTDL1A is therefore the only known candidate to be the rice orthologue of *MAC1*.
2.2 PC, A	Isolated rice orthologues of *Arabidopsis* CLV, WUS, STM, and AG genes	1. Structural orthologues identified and completed. 2. Conservation of function by orthologues confirmed, not confirmed for OsWUS.	Published April 2007 (Rico Gamuyao, MSc thesis)	Structural orthologues of all of these *Arabidopsis* genes were found in the rice genome. However, expression analysis of 11 *OsWOX* genes by RNA *in situ* hybridization established that the *OsWOX* genes most closely related in structure to WUS are not expressed similarly in rice. Since 2.2, 2.5, and 2.6 were conceived as alternative to 2.1, 2.3, and 2.4, the success of 2.1 and the failure of 2.2 led us to abandon 2.5 and 2.6 (approved by external examiners at the mid-term review in August 2006).
2.3 PC	System to control genes of nucellus from megaspore mother cell (MMC1)	1. Demonstration that OsTDL1A secreted from MMC1 controls nucellar cells by interacting with MSP1. This interaction was confirmed but RNA *in situ* hybridization failed to confirm the role of MMC1. 2. Swapping of MSP1 kinase domain to allow MMC1 to control gene expression in nucellus was not pursued because of earlier result.	Published May 2008 (see Zhao et al)	RNA *in situ* hybridization data suggest that expression patterns of OsTDL1A and MSP1 cannot explain the location of cells that form secondary megaspore mother cells (MMC2s). A third component of the system likely remains to be identified.
2.4 PC	OsMac1-based system for inducing second MMC (MMC2)	RNA interference targeted against OsTDL1A induces multiple MMC2s, confirmed.	Published May 2008 (see Zhao et al)	OsTDL1A-RNAi lines showed multiple MMC2s in ovule. System heritable, stable, and male-fertile.
2.5 PC	WUS-induced FI embryogenesis in MMC2		Terminated as unnecessary	See comments on 2.2
2.6 PC	WUS-induced FI embryogenesis in nucellar cluster proximal to MMC1		Terminated as unnecessary	See comments on 2.2

PC = partner country, A = Australia

Objective 3: Apomictic hybrid rice

no.	activity	outputs/ milestones	completion date	comments
3.1 PC, A	Line displaying FI formation of embryo, endosperm, and pericarp		Postponed until Phase 3	This activity depended on the availability of separate rice lines displaying FI embryo, FI endosperm, and FI pericarp and seed coat. None of these lines was convincingly produced and could therefore not be crossed to complete this activity.
3.2 PC, A	Line in which endosperm feeds apomictic embryo during germination		Postponed until Phase 3	This activity depended on successful completion of 3.1
3.3 PC, A	One-line apomictic hybrid rice		Postponed until Phase 3	This activity depended on successful completion of 3.2

PC = partner country, A = Australia

Objective 4: Communication and dissemination of research results

no.	activity	outputs/ milestones	completion date	comments
4.1 PC, A	Publications and Web site for pre-publication releases	Bi et al (2005) Gamuyao (2007 thesis) Zhao et al (2008) Bennett & Zhao (2008)	Web site replaced by FTP for file transfers	Progress on this project was not such that prepublication releases or postpublication postings were feasible until almost the end of the project.
4.2 PC, A	Participation in Third International Symposium on Apomixis	1. Paper by Bennett 2. Paper by Luo	June 2007	Completed
4.3 PC, A	Mid-term review in Canberra	1. Reports by Bennett and by Luo 2. Review by Xie	August 2006	Completed
4.4 PC	Participation in Fourth International Congress on Hybrid Rice, Changsha, China	1. Bennett, invited speaker	September 2008	Dr. Bennett was unable to participate because of another commitment.
4.5 PC, A	Terminal workshop at IRRI	1. Reports by Bennett and by Luo 2. Reviews by A. Koltunow (CSIRO), I. Siddiqi (India), I. Slamet-Loedin (Indonesia), Y.-Z. Xing (China)	April 2008	Completed
4.6	International Epigenome Conference, Blue Mountain, Australia	Luo, invited speaker	September 2008	In preparation

PC = partner country, A = Australia

7 Key results and discussion

At the end of Phase 1 of this project (1997-2002), CSIRO reported that sexuality and apospory in *Hieracium* are interrelated developmental pathways that differ principally at the start and the end (Tucker et al 2003). The main difference at the start of the pathway is the occurrence of meiosis in sexual plants compared with the initiation of apospory in apomicts. In Phase 2, IRRI has focused on switching off meiosis and switching on apospory in rice. A major difference at the other end of the pathway was fertilisation dependency vs. autonomy in endosperm formation. In Phase 2, CSIRO has studied this switch in rice. Here, we report and discuss key results in these two areas.

7.1 A two-step approach to producing aposporous initials (AIs) in rice

AIs often arise from nucellar cells of the ovule. The AIs are located adjacent to the MeMC or the megaspore (Bicknell and Koltunow 2004). IRRI hypothesized that AIs may be related to the additional MeMCs that form in the nucellus when the genetic control over MeMC numbers is relaxed, as first shown in the multiple archesporial cells1 (*mac1*) mutant of maize (Sheridan et al 1996). Like the MeMC, AIs are large nucellar cells that have an ability to give rise to embryo sacs—diploid in the case of apospory, haploid in the case of sexuality. The important difference from MeMCs is that AIs do not enter meiosis; they just undergo mitosis to form embryo sacs. Later work established that mac1 also caused extra meiocytes to form in the anther (Sheridan et al 1999). IRRI's objective was therefore (i) to identify the rice orthologue of the *MAC1* gene and to down-regulate it using RNA interference (RNAi) to produce additional MeMCs in the ovule, and (ii) to bypass meiosis in those additional MeMCs in the expectation that they would initiate aposporous embryo sac formation.

7.1.1 The search for the rice orthologue of *MAC1* in maize

Although maize *MAC1* has still not been isolated from maize, identifying the rice orthologue did not appear to be a major problem because studies in *Arabidopsis* had identified mutants that phenocopied *mac1* in the anther if not in the ovule (Canales et al 2002, Zhao et al 2002, Yang et al 2003, 2005). Unlike *mac1*, which had been produced using Robertson's mutator, these *Arabidopsis* mutants had been produced by insertional mutagensis that tagged the genes and permitted their isolation. Cloning of the genes, named *EXS*, *EMS1*, and *TPD1*, revealed that the *exs* and *ems1* mutants (Canales et al 2002, Zhao et al 2002) occurred in the same gene, which encoded a large leucine-rich repeat receptor kinase, while the *tpd1* mutant (Yang et al 2003, 2005) occurred in a different gene that encoded a small protein that was later hypothesized to be an extracellular ligand of *EXS/EMS1* (Ma 2005).

A major advance occurred at the start of Phase 2 when Nonomura et al (2003) discovered the rice gene *MULTIPLE SPOROCYTES1* and showed that three *msp1* mutants phenocopied the *mac1* mutants in both anther and ovule. The mutant did not appear to result in the formation of unreduced embryo sacs, to judge from the limited amount of progeny examined. The *Tos17* insertion events that created *msp1* mutants tagged the gene and allowed its identification as a leucine-rich repeat receptor kinase that turned out to be the orthologue of *EXS/EMS1*. However, knockout mutants such as *mac1* and *msp1* have the disadvantage that their phenotype in the anther includes male sterility, a trait that would limit the utility of these mutants in developing apospory in hybrid rice. IRRI and CSIRO had envisioned isolating a set of genes required for apospory and distributing them between the male and female parents of hybrid rice in such a way that the two parents would be self-fertile but their hybrid would be apomictic. The number of genes required for apospory is unclear but Matzk et al (2005) discovered that five genetic loci

distinguished the sexual and aposporous lines of Kentucky Blue Grass (*Poa pratensis*). Three of the five genes (*apv*, *AIT*, *mdv*) are involved in the switch from normal sexuality to the production of an aposporous initial, while the remaining two genes (*ppv*, *PIT*) are involved with parthenogenesis for the production of the apomictic embryo. In *P. pratensis*, the endosperm is formed by pollination and fertilisation of the central cell of the unreduced embryo sac. Matzk et al (2005) suggested that *apv* might be the *Poa* orthologue of *MAC1*. If five genes are required for apospory in rice, IRRI and CSIRO would plan to introduce perhaps three of them into one parent and the other two into the second parent without jeopardizing sexual reproduction in either parent. Male sterility would prevent this approach from being successful.

Accordingly, IRRI began to apply RNAi to this problem in the hope that it would be possible to choose a promoter that would activate the interference mechanism in the ovule but not in the anther (knock-outs caused by Robertson's mutator, *Tos17*, or T-DNA do not generally show this discrimination). Three genes were targeted by RNAi: *MSP1* and two putative rice orthologues of TPD1, which we have named *OsTDL1A* and *OsTDL1B* (Zhao et al 2008). It is possible that *MAC1* in maize is actually ZmTDL1A or ZmMSP1 or the maize orthologue of any other protein that interacts with *MSP1* and *OsTDL1A* to control meiocyte numbers, but this remains to be determined.

7.1.2 Successful identification of OsTDL1A as a ligand of *MSP1*

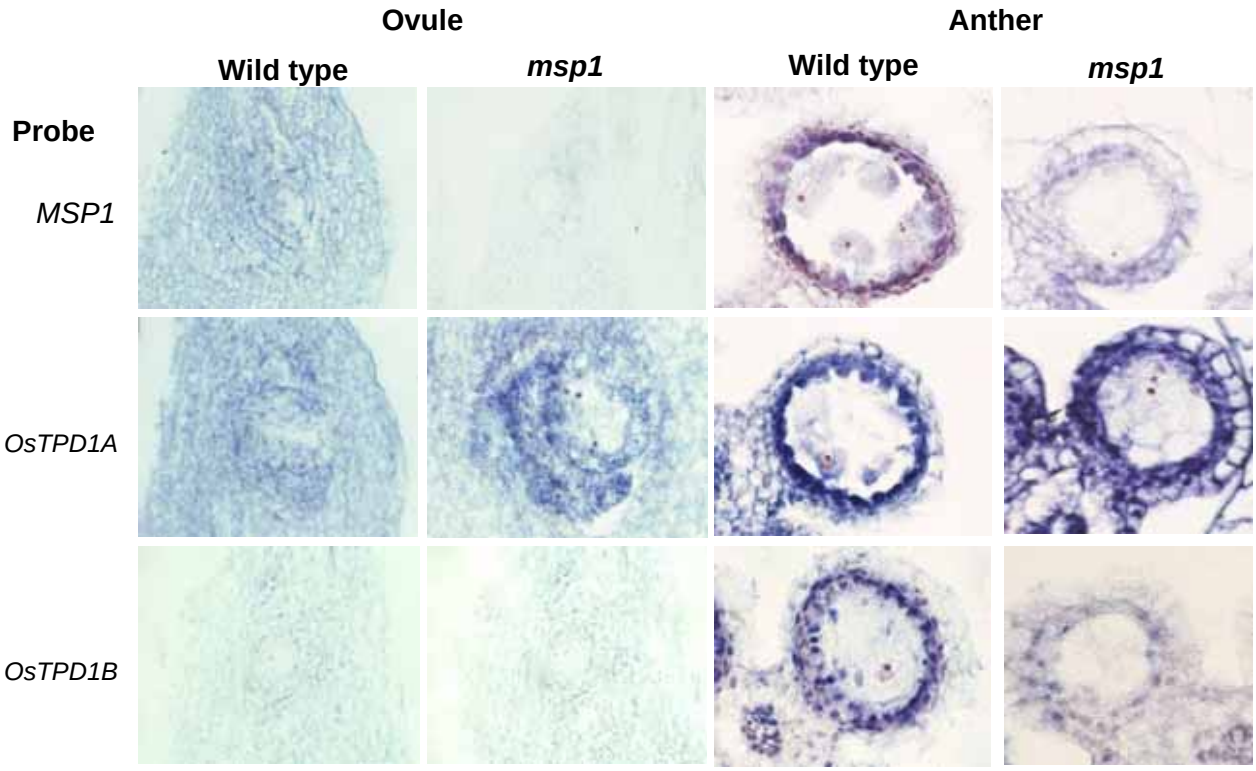

Fig. 1. RNA in situ hybridization of antisense probes for MSP1, OsTPD1A, and OsTPD1B in ovules and anthers of 3-mm-long spikelets of wild-type and homozygous msp1 mutant plants.

Although OsTDL1A and OsTDL1B are the rice proteins closest in sequence to TPD1 of *Arabidopsis*, we noted that OsTDL1A was a closer relative to TPD1 than was OsTDL1B. However, both OsTDL1A and OsTDL1B were co-expressed with *MSP1* in rice florets at the meiosis stage as judged by RT-PCR. When we used RNA *in situ* hybridization to establish more precisely the sites of expression of these genes, we found that *OsTDL1A* and *MSP1* were co-expressed in the anther and the ovule, whereas *OsTDL1B* was co-expressed with these genes only in the anther (Fig. 1). Furthermore, in collaboration with CSIRO, IRRI used the yeast two-hybrid system and bimolecular fluorescence

complementation in onion cells to show that it is *OsTDL1A* rather than *OsTDL1B* that has the ability to act as a ligand to the extracellular LRR domain of *MSP1* (Zhao et al 2008).

7.1.3 Successful induction of additional MeMCs in rice through RNAi

We used RNAi targeted against *OsTDL1A* to investigate whether down-regulation of *OsTDL1A* expression would phenocopy *msp1*. We found that *OsTDL1A*-RNAi lines phenocopied *msp1* in the ovule but not in the anther (Zhao et al 2008). Multiple MeMCs were present in the ovule of *OsTDL1A*-RNAi lines (Fig. 2) but male sterility was not observed. This was a very useful result because *msp1* is a difficult mutant with which to work; it is a recessive lethal mutation that must be carried as the heterozygote. The fertility of the *OsTDL1A*-RNAi lines makes them much easier to use in crosses to pyramid genes required for synthetic apospory.

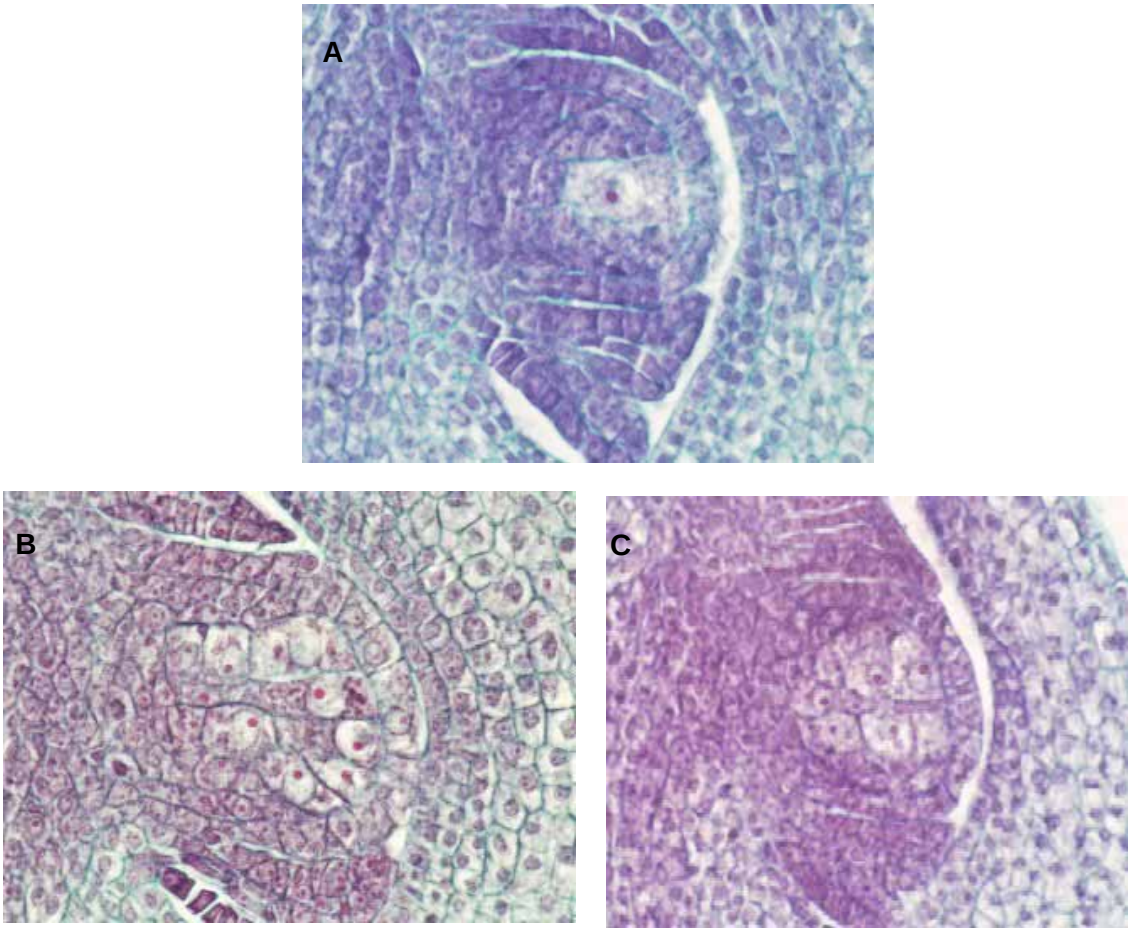

Fig. 2. RNAi of OsTPD1A gene phenocopies homozygous msp1 mutant in inducing multiple megaspore mother cells in the rice ovule. (A) Wild-type cultivar Nipponbare. (B) Homozygous msp1 mutant. (C) RNAi-OsTPD1A line 4363.

7.1.4 Why are *OsTDL1A*-RNAi lines male-fertile?

We sought the reason for the absence of male sterility in *OsTDL1A*-RNAi lines. One possible explanation derives from the fact that the RNAi cassette used in this work is expressed under the control of the maize ubiquitin1 promoter (Miki and Shimamoto 2004). This promoter is not well expressed in the tapetum of the rice anther (Cornejo et al 1993), a major site of expression of *OsTDL1A* in the anther. In fact, considerable *OsTDL1A* expression occurs in the tapetum of RNAi plants, although at a lower level than seen in the nontransformed control. Our conclusion is therefore that RNAi is insufficiently effective in down-regulating *OsTDL1A* transcription in the tapetum to produce a phenotype. We

also examined whether the presence of a heat-shock element in the ubiquitin1 promoter might allow high temperatures to enhance down-regulation in the anthers of *OsTDL1A*-RNAi plants and so induce male sterility. We found that exposure of the RNAi lines to 40 or 45 °C had only a slight effect on the expression of the cassette, whereas exposure to 45 °C had a major inductive effect on endogenous heat-shock genes in RNAi lines and the nontransgenic control but caused sterility only in the RNAi lines (on-line supplementary material in Zhao et al 2008). We conclude that, in the field, where a temperature of 45 °C would not be expected, the *OsTDL1A*-RNAi lines would remain male-fertile.

7.1.5 Is the induction of multiple MeMCs by *OsTDL1A*-RNAi lines stably heritable?

We focused on *OsTDL1A*-RNAi line #4363 and established that the RNAi cassette was transmitted in a functional form to the T1 and T2 generations, both of which exhibited multiple MeMCs in the ovule and were fertile, with no sign of a phenotype in the anthers (Zhao et al 2008). Co-segregation was observed between the presence of the RNAi cassette and the ability to produce multiple MeMCs. We conclude that this *OsTDL1A*-RNAi line is an effective and convenient platform from which to develop apospory in rice.

7.1.6 Is the *OsTDL1A*-RNAi cassette effective in indica backgrounds?

The initial study reported above was conducted on japonica cv. Nipponbare, but most hybrids are formed in indica backgrounds. To study the effectiveness of the *OsTDL1A*-RNAi cassette in indica backgrounds, cvs. Kasalath and IR64 were transformed with an *OsTDL1A*-RNAi plasmid and a cassette-free control plasmid. T0 plants were recovered (>40 for Kasalath and >30 for IR64) and T1 seeds were harvested. All putative transformants for Kasalath and IR64 were found to contain the RNAi cassette as judged by PCR assays. Ovules of T0 Kasalath transformants were positive for the *OsTDL1A*-RNAi cassette, all of which showed down-regulation of *OsTLD1A* transcripts with no interference with expression of *OsTDL1B* or *MSP1*. Microscopy established that multiple large MeMC-like cells are indeed present in the ovules of the Kasalath transformants. T1 plants are now being grown to test for inheritance and functionality of the RNAi cassette.

7.1.7 Is meiosis in the secondary MeMCs different from that in the primary MeMCs?

As part of their effort to bypass meiosis in secondary MeMCs, IRRI investigated whether meiosis in the secondary MeMCs of the *msp1* mutant differs from meiosis in the primary MeMC of wild-type plants. IRRI examined the expression of six rice genes representing homologues of activators and inhibitors of the anaphase-promoting complex/cyclosome (APPC/C). In yeast and animals, APC/C is an E3 ubiquitin ligase that drives the breakdown of specific cell-cycle proteins in response to the binding of activators such as CDC20 and CDH1 and inhibitors such as RBR. IRRI found that rice contains two homologues of each of these proteins. Using RNA *in situ* hybridization, IRRI established that CDC20A, CDC20B, and RBR1 are strongly expressed in mitotic and meiotic cells of the ovule and anther, whereas CDH1A, CDH1B, and RBR2 are preferentially expressed in mitotic cells. They found little or no difference between primary and secondary MeMCs with respect to expression of these genes. In no case did they see an RNA *in situ* hybridization signal with sense probes, that is, they detected no antisense transcripts for these genes.

7.1.8 How is meiosis initiated in rice?

To bypass meiosis in secondary MeMCs, IRRI assumed that it would be necessary to block an early step in its initiation. The reasoning is that to preserve the genotype of the hybrid in aposporous offspring, the secondary MeMCs must be diverted away from meiosis and toward diploid mitosis before either recombination or segregation occurs. As both of these events occur during meiosis I, it would be necessary to divert the cells either

before or immediately after premeiotic S-phase but certainly before the dyad cell stage, which marks the completion of meiosis I. However, a recent report on the dyad mutant of *Arabidopsis* (Ravi et al 2008) suggests that, at a low frequency, the MeMC can divide to make a dyad by going through an unusual form of mitosis rather than meiosis I. Whether this frequency can be increased and also reproduced in rice is unclear. Accordingly, IRRI continues to identify an early step in the initiation of meiosis as a possible intervention point.

Although the details of meiosis I and meiosis II are highly conserved across fungi, animals, and plants, the details of the initiation of meiosis are highly variable. The initiation process is most thoroughly studied in budding yeast and fission yeast and differs markedly between these two divergent fungi. IRRI searched the rice genome for homologues of genes required for the initiation of meiosis in both types of yeast and found a high degree of conservation in budding yeast. There was no homologue for IME1, a key transcription factor, and no close homologue for IME2, a protein kinase. However, a close homologue was found for IME4, an mRNA N6-adenosine methyltransferase (Clancy et al 2002). Significantly, we found that OsIME4 sense transcripts accumulated in mitotic cells, whereas OsIME4 antisense transcripts accumulated in meiotic cells (Fig. 3). This was true for ovules and anthers and for wild-type and *msp1* mutant genotypes. Intriguingly, sense/antisense transcripts of IME4 control meiosis in budding yeast (Hongay et al 2006). IRRI is now exploring the possibility of using overexpression and RNAi to alter the ratio of sense and antisense transcripts for OsIME4.

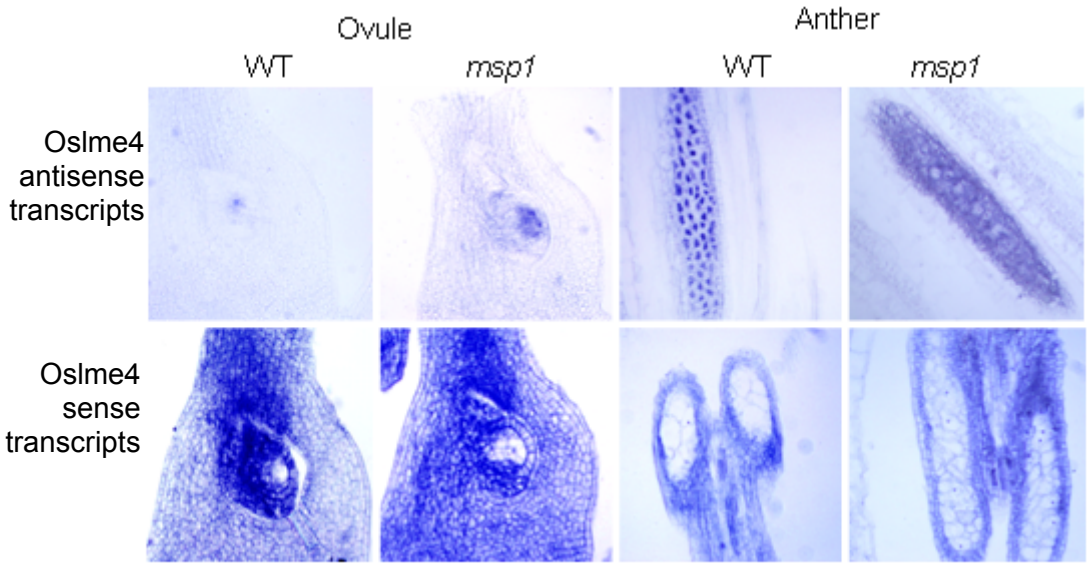

Fig. 3. RNA in situ hybridization shows the presence of sense and antisense transcripts of OsIME4 in the anthers and ovules of wild-type and msp1 mutant. The sense transcripts are located in mitotic cells while the antisense transcripts are located in premeiotic cells.

7.2 The function of OsFIS Polycomb Group genes in rice

Although FI endosperm formation is not essential for constructing apomixis in rice, it has several important advantages over fertilisation-dependent endosperm formation, including easier passage through regulatory procedures on account of the absence of transgenic pollen. The proposed method for FI formation of the endosperm in rice involved the down-regulation of rice counterparts of fertilisation-independent seed (FIS)-like genes based on the prior Phase 1 analyses of endosperm induction in *Arabidopsis FIS* mutants. In Phase 1, the three then-known *Arabidopsis FIS* genes were named *FIS1* or *MEA*, *FIS2*, and *FIS3*. These genes function as Polycomb Group genes to modulate chromatin function. They are now known to fall into functional classes of genes called E(z), VEFS, and ESC homologues, respectively, when compared with other plant and animal species. Using the *Arabidopsis* sequences of E(z), VEFS, and ESC homologues as queries, we searched the indica and japonica genomic sequence databases available from Gene Bank and Rice Functional Genomic Express (http://signal.salk.edu/cgi-bin/RiceGE) to identify all of the FIS-like genes in rice. Phylogenetic analysis compared these sequences to all others currently known in plants and was carried out to determine those most likely to perform E(z), VEFS, and ESC function. All of the candidates were down-regulated in rice using RNAi. Expression of all genes was examined by fusing them to a colorimetric reporter. All of these analyses involved the generation of a minimum of 40 independent transgenic lines per gene construct. Imprinting analysis was also carried out on the endosperm because FIS Polycomb Group gene function in *Arabidopsis* involves parental genome-specific imprinting during seed development.

7.2.1 Rice has two FIS1 or E(z) homologues

Although there are three E(z)-like genes [FIS1/MEA, CLF, and SWN(SWINGER)] in the *Arabidopsis* genome, we found two E(z)-like genes in the rice genome, Os03g19480 on chromosome 3 and Os06g16390 on chromosome 6. Os03g19480 is identical to two cDNA sequences designated with different names: OsiEZ1 (Aj421722) and OsSET1 (AF407010) (Liang et al 2003, Thakur et al 2003). Os06g16390 is also identical to cDNA sequences (AK111743 and J023052J10). In BLAST searches, the Os06g16390 gene has highest similarity to CLF, so we designated it as OsCLF.

A phylogenetic tree analysis, using the E(Z)-like sequences from 50 taxa, shows that an SWN clade exists that includes two sub-clades: the dicot SWN-like proteins and the monocot SWN-like proteins. OsiEZ1 (Os03g19480) grouped with Mez2 and Mez3 in the monocot SWN-like sub-clade. MEZ2 and MEZ3, which share 89% sequence identity, are likely to be genome duplicates resulting from the paleotetraploid origin of maize (Springer et al 2002). *Arabidopsis* SWN groups with other dicot SWN-like proteins. The CLF-like protein clade includes proteins from spike-moss, monocots, and dicots. The separation between CLF and SWN lineages happened at least before the divergence of monocots and dicots. Lower plants (Physcomitrella and Silaginella) have a single CLF homologue. SWN may have originated within higher plants. Rice CLF-like protein (Os06g16390) was found to be more related to Mez1 than to other CLF-like proteins from other species.

FIS1/MEA was found to group with other FIS1/MEA-like proteins from *A. arenosa* and *Brassica*, indicating a distinct evolutionary origin. No FIS1/MEA-like protein has been identified from other species except in Brasicaceae. Recent results showed that FIS1/MEA is duplicated from SWN as a result of a whole-genome duplication within the Brassicaceae lineage. Under positive selection, FIS1/MEA function had become specific to seed development in *Arabidopsis*, whereas SWN had evolved under purifying selection (Spillane et al 2007).

We further examined whether rice E(Z) homologues contain all of the domains conserved in other widespread E(Z)-like genes. We made sequences alignments of E(Z) homologues of rice, *Arabidopsis*, and *Drosophila* (Fig. 2). A total of five domains, EZD1, EZD2, SANT, Cys-rich, and SET, were found. Similar domains have been found in all three maize E(Z)

homologues (Springer et al 2002). The SET domain is involved in histone H3 lysine27 tri-methylation (Rea et al 2000, Cao et al 2002, Czermin et al 2002, Kuzmichev et al 2002, Müller et al 2002). SANT domains are often involved in nonspecific DNA binding (Aasland et al 1996) and the functions of the other domains are not known.

7.2.2 Rice has two homologues containing FIS2-like (or VEFS domain) proteins

There are four VEFS domain-containing proteins in *Arabidopsis*: FIS2, VRN2, EMF, and AT4G16810 (reviewed by Pien and Grossniklaus 2007). However, there are only two similar proteins, Os09g13630 and Os04g08034, in rice, and both had been assigned the same name, OsEMF2 (http://signal.,salk.edu/cgi-bin/RiceGE). To avoid confusion, we designate Os09g13630 as OsEMF2a and Os04g08034 as OsEMF2b. OsEMF2b is associated with several cDNA sequences that result from alternative splicing (http://signal.,salk.edu/cgi-bin/RiceGE).

Phylogenetic tree analysis indicated that there is a main EMF2 clade including lower and higher plants. *Arabidopsis* EMF2 was grouped with other dicot EMF2-like proteins, while the two rice EMF2-like proteins were grouped with other grass EMF2-like proteins. Among the higher plants, dicots and the basal monocot groups have a single homologue of EMF2, but rice, barley, and maize all have two homologues of EMF2. VRN2-like proteins form a group within Brassicaceae, which are duplicates and have diverged significantly from the main EMF2 clade. We further investigated whether the FIS2 is duplicated from VRN2 or EMF2 in *Arabidopsis*. FIS2 (chr. 2, 15033493–15000348 bp) was duplicated from the VRN2 region (chr. 4, 9291141–9476162bp) in the *Arabidopsis* genome (Blanc et al 2003, Simillion et al 2002, Grant et al 2000, www.tigr.org/tdb/e2k1/ath1/Arabidopsis_genome_duplication.shtml), suggesting that FIS2 might have undergone a similar evolutionary process as FIS1/MEA, which arose from a block duplication of the SWN region (Spillane et al 2007). FIS2 appears to be the only gene that had been specified to have a function in seed development in *Arabidopsis* because we observed no seed phenotype in *emf2* and *vrn2* mutants. At4g16810 formed an out-group related to other VEFS proteins in plants. We did not identify any VRN2-like and FIS2-like proteins outside of the Brassicaceae using these analyses.

Sequence alignments of the VEFS homologues of rice, *Arabidopsis*, and *Drosophila* were made. The sequence alignments indicated that there are zinc-finger motifs and VEFS domains conserved in all proteins except At4g16850, which does not contain a zinc finger.

7.2.3 Rice has two FIS3 (or ESC homologues)

In contrast to the existence of only one copy of the ESC homologue, FIS3/FIE in *Arabidopsis*, there are two rice ESC homologues, Os08g04270 and Os08g04290. These two genes are closely located together on chromosome 8 with one gene separating them. Maize also has two *FIS3/FIE* genes. Os08g04270 is highly homologous to maize *ZmFie2* and thus was designated as OsFIE2. Os08g04290 was designated as OsFIE1. The amino acid sequences of OsFIE2 and OsFIE1 are 72% identical to each other over their entire length. *FIE* is 69% identical to OsFIE2 and 66% to OsFIE1 over their entire lengths. Sequence alignment indicates that there are seven conserved WD40 repeats typical of this protein group.

The phylogenetic analysis of the ESC genes clearly showed two major clades that correspond to Metazoans and plants, with the exception of *Suberites* and *C. elegans*, which are out-grouped. In the plant clade, the grasses (maize, *Sorghum*, and rice) have two copies of FIE-like genes. The dicot clade includes several species, each of which has one copy of the FIS3/FIE homologue.

7.2.4 Expression analysis of rice Polycomb genes

We characterized the temporal and spatial expression patterns of the two rice VEFS genes, the two rice E(Z)-like genes, and the two FIS3/FIE-like genes using reverse

transcriptase-mediated (RT) PCR with total RNA as a template (Fig. 4). Total RNA was extracted from stems, leaves at flowering, young leaves at the seedling stage, anthers prior to their opening (dehiscence), immature panicles, roots, whole seeds 2 days after fertilisation, and endosperm extracted from seeds at 4 and 9 days after fertilisation. *OsFIE1* was found to be an endosperm-specific gene. It was detected in the endosperm 4 and 9 days after fertilisation. All other tissues examined did not express *OsFIE1*, indicating that its expression is tightly regulated. By contrast, OsFIE2 mRNA was detected in all tissues examined. A similar observation has been made for the two maize *FIS3/FIE* genes: *ZmFie1* shows endosperm-specific expression, while *ZmFie2* is detected in various tissues (Springer et al 2002, Denilevskaya et al 2003). *OsiEZ1*, *OsCLF*, *OsEMF2a*, and *OsEMF2b* genes were expressed in all the tissues tested. These data were also confirmed using colorimetric *GUS* gene fusions in transgenic rice plants.

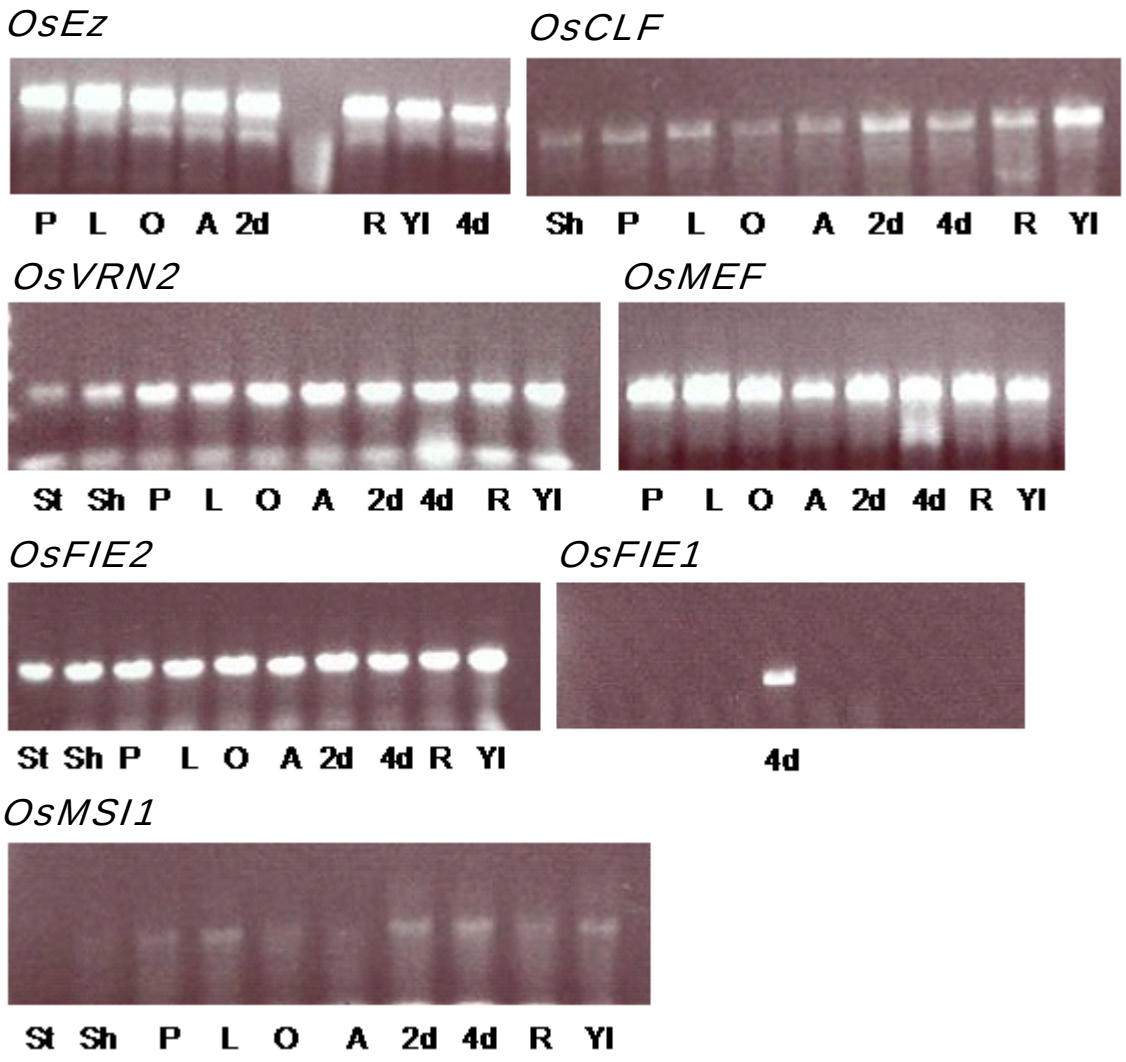

Fig. 4. RT-PCR expression of rice FIS-class genes in different tissues. St: stem; Sh: leaf sheath; P: young panicle; L: leaf; O: ovule; A: anther; 2d: 2-day-old seed; 4d: 4-day-old endosperm; R: roots; Yl: young leaf.

7.2.5 Parental expression of Polycomb genes in endosperm

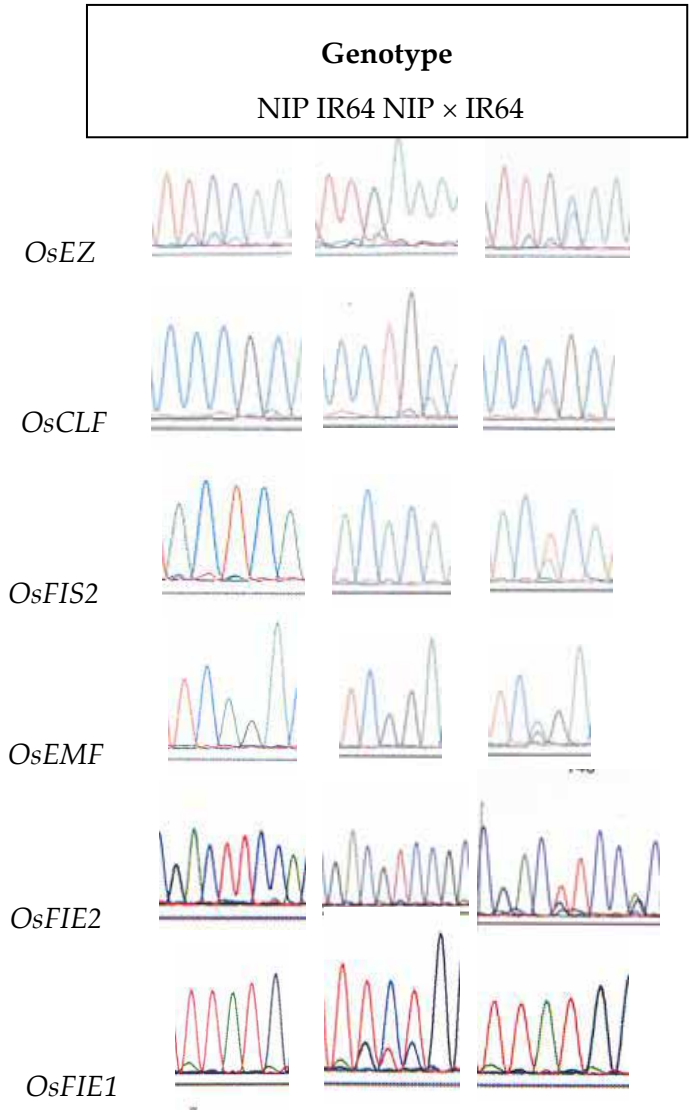

Fig. 2. Parental expression of Polycomb genes in endosperm of a Nipponbare × IR64 cross.

The *Arabidopsis* FIS polycomb genes *MEA* and *FIS2* are imprinted, that is, there is differential expression of the genes from maternally and paternally inherited genomes (Luo et al 2000, Kinoshita et al 1999, Vielle-Calzada et al 1999). The maize polycomb genes *ZmFie1* and *Mez1* are also imprinted (Denilevskaya et al 2003, Haun et al 2007). To better understand the function of the different polycomb genes in rice, we investigated the imprinting status of these genes using hybrid endosperm formed following a cross between a japonica rice line (Nipponbare) and an indica rice line (IR64). Total RNA was extracted from the endosperm of seeds after fertilisation of Nipponbare with IR64 pollen and also seeds from the self-fertilized parental lines. RT-PCR was conducted using primers selected to cover polymorphisms between parental lines. Thus, the primers could not only detect the gene of interest but also indicate whether the amplified products after sequencing were from the maternal or paternal genomes or from both genomes. When the expression of *OsEZ1*, *OsCLF*, *OsEMF2a*, *OsEMF2b*, and *OsFIE2* genes was examined in hybrid endosperm and compared with that from both parents, it was clear that the sequences representing gene expression from both parents were evident (Fig. 2).

Following further quantitative analyses, we found that that the hybrid endosperm contained mRNAs from both parents in a ratio close to 2 maternal copies to 1 paternal copy. Endosperm in rice comprises two maternal genome equivalents to one paternal genome equivalent. Therefore, the result clearly indicates that both maternal and paternal copies of the same gene were equally expressed in the triploid endosperm. However, *OsFIS3/FIE1*, the rice endosperm-specific gene, was found to be imprinted in the hybrid endosperm of Nipponbare pollinated with IR64. Sequencing of the PCR products from the hybrid endosperm confirmed that the paternal message was completely absent in the hybrid endosperm and all of the message detected came from the maternal genome. This result was confirmed in a reciprocal cross in which IR64 was pollinated with Nipponbare. Imprinting is also observed in ZmFie1, which is the maize orthologue of OsFIS3/FIE1.

7.2.6 Down-regulation of *OsFIS* genes and the analysis of T-DNA insertion mutants to test for the induction of fertilisation-independent endosperm in rice

In order to investigate whether down-regulation of *OsFIS* gene function in rice or direct mutagenesis of rice *OsFIS* genes would induce fertilisation-independent endosperm development, we used RNAi to down-regulate the genes and also examined T-DNA mutants carrying insertions in rice *OsFIS* genes. We obtained T-DNA insertion lines in *OsCLF*, *OsFIS3/FIE1*, and *OsEMF2a*. All of the insertion lines were screened to confirm the presence and to define the location of the T-DNA insertion in these genes and homozygous T-DNA insertion lines were identified for all except *OsFIS1/FIE* (discussed below). In *Arabidopsis*, mutations in *CLF* induce a leaf phenotype but we did not observe any obvious vegetative phenotypes in the homozygous *OsCLF* T-DNA insertion mutant.

Homozygous *OsFIS3/FIE1* plants could not be generated. Even though 150 plants derived from a heterozygous T-DNA insertion mutant were screened, only wild-type plants and plants heterozygous for the T-DNA insertion were obtained. In *Arabidopsis*, plants heterozygous for At FIS3/FIE display 50% aborted seeds following pollination. However, the heterozygote OsFIE1/Osfie1 set fully developed seeds at a ratio comparable to that of the wild type and all of the seeds germinated. Reasons for the inability to generate a homozygous *OsFIS3/FIE1* T-DNA mutant line were not clear and the analysis was not pursued further because of the functional observations in unpollinated plants described below.

T-DNA insertion in the *OsEMF2a* gene did not cause an obvious phenotype in the vegetative plant but the plants flowered earlier than the wild type when both were grown under long-day conditions (16 h). At 120 days after germination, panicles could be seen in the mutant, while the wild type remained at the vegetative stage. Most panicles did not elongate sufficiently to be seen outside the sheath. On average, only 1 or 2 panicles in each mutant plant with about 10 tillers might protrude half way outside the sheath and spikelet development was arrested when anthers and pistils had differentiated and then most spikelets etiolated. Occasionally, some spikelets had multiple ovaries. Each panicle in the mutant had 20.5 ± 5 aborted spikelets while wild-type plants developed about 41 ± 7 fertile spikelets per panicle. When both the mutant and the wild-type plants were grown under short-day conditions, both flowered around the same time. These phenotypes co-segregated with the T-DNA insertion in homozygous plants in a segregating population. The wild-type *OsEMF2a* appears to function in the repression of flowering and in the promotion of regulating normal, fertile spikelet development in rice under long-day growth conditions.

The T-DNA insertion lines of *OsEMF2a*, *OsFIE1*, and *OsCLF* were also emasculated to investigate whether the mutants would produce autonomous endosperm as observed in *Arabidopsis FIS*-class mutants. Surprisingly, all of the plants in segregating populations of the *OsEMF2a*, *OsFIE1*, and *OsCLF* T-DNA mutants were able to form autonomous seed-like structures regardless of the presence or absence of the T-DNA insertion. The development of autonomous seed-like structures was delayed in emasculated plants.

Obvious seed elongation was observed 3 days after pollination but autonomous seed-like structures initiated only 10–15 days after emasculation. These autonomous structures could attain the size of a fully developed seed but they then collapsed. Dissection of these expanded autonomous structures indicated that they contained a clear liquid, rather than the milky endosperm observed in fertilized seeds. Autonomous and developing fertilized seeds were stained with an iodine solution to detect the presence of starch and this was present only in the endosperm of fertilized seeds. We sectioned the autonomous seed-like structures and also the fertilized seeds at different stages of development. Fertilized seeds contained cellularized endosperm surrounded by proliferating maternal cells. However, in the autonomous seeds, an excess of proliferating maternal cells could be seen surrounding a collapsed embryo sac. Evidence for autonomous endosperm formation was not evident in the autonomous seed-like structures.

Unfortunately, autonomous endosperm formation as seen in *Arabidopsis* has not been observed in any of the more than 240 transgenic plants containing RNAi constructs down-regulating the *OsFIS*-class genes. Autonomous seed-like structures did form following emasculation. We found that the frequency of formation of these autonomous seed structures was highly variable between plants and within plants. In general, panicles from one plant showed different levels of autonomous seed-like set from 0% to 30%. Some plants displayed no autonomous seed set. The sections of autonomous rice seed-like structures very much resembled the "pseudo-seeds" found in parthenocarpic tomato fruits when auxin signal transduction and auxin response factor 8 function is altered (Goetz et al 2007). It may be that, in rice, emasculation is able to trigger or uncover a low level of hidden parthenocarpic response in some rice genotypes, which leads to the formation of a pseudo-seed.

In summary, phylogenetic tree analysis indicates that the rice genome does not possess orthologues of *MEA* and *FIS2*. Both *MEA* and *FIS2* arose from a comparatively recent gene duplication in *Arabidopsis*. Rice does contain other genes homologous to those of the *FIS*-class in *Arabidopsis*. However, functional analysis in rice by down-regulation and mutation indicates that they are not essential for preventing endosperm formation in the absence of fertilisation in rice. Recent analysis in *Hieracium* has also indicated that the FIS3/FIE-like gene is not essential for repressing endosperm initiation in the absence of fertilisation in sexual species but it is essential for the early events of embryo and endosperm formation in both sexual and apomictic species (Rodruiges et al 2008). Maize *FIE1* and *FIE2* do not appear to be required to repress central cell proliferation (personal communication with Dr. Jose Gutierrez-Marcos). Rice may have a different mechanism regulating endosperm initiation and this needs investigation.

7.2.7 Analysis of the efficacy of autonomous embryo induction in rice in At MSI T-DNA mutants

The *Arabidopsis MSI1* gene is also a chromatin remodelling protein of the Polycomb Group. It appears to be involved in the control of seed initiation in *Arabidopsis* as mutants in *MSI1* undergo fertilisation-independent endosperm proliferation and, surprisingly, also initiate fertilisation-independent enbryogenesis. The seeds are not viable. We attempted to examine the role of rice *MSI1* in seed development by obtaining rice lines with T-DNA insertions in OSMSI1-like genes from Taiwan. Unfortunately, the putative T-DNA lines established in the glasshouse from Tiawan to date have failed to contain T-DNA inserts in rice *MSI1* genes following molecular analysis and they have not been pursued further.

8 Impacts

8.1 Scientific impacts—now and in 5 years

Science progresses through the development and application of powerful new technologies to rigorously test clear hypotheses. This project has applied the new technique of RNA interference (RNAi) to rice to test two hypotheses: (i) the control of megaspore mother cell (MeMC) numbers in the rice ovule is mediated by the same receptor-ligand mechanism as operates in *Arabidopsis*, and (ii) induction of autonomous endospermy in rice ovules will be achieved by inactivating the FIS-polycomb complex as in *Arabidopsis*. Our data provide strong support for the first hypothesis but reject the second. One outcome of *FIS*-class gene analysis in rice was that one of the genes, *OsEMF2a*, appears to function in the repression of flowering and in the regulation of normal, fertile spikelet development in rice under long-day growth conditions. There may be applications of this finding to rice breeding. Our success with the control of MeMC numbers may encourage other scientists to use *OsTDL1A*-RNAi lines as a platform to develop synthetic apomixis in rice and to explore the operation of the receptor-ligand complex in natural apomicts. By contrast, our RNAi and knock-out data on the FIS-Polycomb genes of rice will encourage scientists to take new approaches to autonomous endospermy in plants other than *Arabidopsis* and especially in cereals. The data presented underscore the need to study processes regulating and controlling rice seed formation. We expect that, within the next 5 years, we shall have a much clearer picture of the diversity of the controls on sporocyte numbers and autonomous endospermy. However, the greatest scientific impact will come from the general encouragement of research on the genes controlling apomixis in natural systems, their use, and testing in rice, and also parallel approaches toward synthetic apomixis in rice as we have begun in case the genes from apomicts are naturally adapted to function in the species from which they are isolated.

8.2 Capacity impacts—now and in 5 years

Team members Dr. Ming Luo (CSIRO), Dr. Xinai Zhao (IRRI), and Mr. Rico Gamuyao (IRRI) have already been accepted by other laboratories to apply their expertise to other systems: Dr. Luo has been hired by the CSIRO laboratory in Adelaide to study the natural apomict *Hieracium*, while Dr. Zhao has moved to Strasbourg to study sexual reproduction in *Arabidopsis* and the apomictic grass *Brachiaria*, and Mr. Rico Gamuyao has been accepted as a PhD student in the Abiotic Stress Laboratory at IRRI to apply his skills in RNA *in situ* hybridization, developed while an MSc student on the project. Technicians Ms. Yi Sao and Min Huang at CSIRO have also been employed in science positions in other organizations.

8.3 Community impacts—now and in 5 years

As this project was conceived as Phase 2 of a three-phase program, we do not expect that it will have economic, social, and environmental impacts by itself, now or in 5 years. These community impacts will depend on the successful completion of Phase 3 (which is now expected to run from July 2009 to June 2014). Our discussion of impacts here is therefore tentative but important in relation to the design of an effective Phase 3.

8.3.1 Economic impacts

The economic impacts of apomictic hybrid rice would arise principally from the higher adoption by farmers of high-yielding hybrids because of lower seed costs and the ability of farmers to reproduce these seeds in their own fields. Phase 3 research must therefore

produce apomictic hybrids that display no yield penalty and that can be produced by manual crossing of apomixis-ready parental lines. These lines must carry complementary sets of apomixis genes while remaining fully self-fertile. The lower seed cost would lead to wider acceptance of hybrids, especially by poor farmers, whose livelihoods would be improved by the higher yield and greater yield stability of hybrids than with inbreds. A second source of economic impact would come from combining apomixis with autonomous endospermy, a situation that would eliminate the need for pollen and thus simplify regulatory approval of most transgenic traits. It is also expected that by-passing the need for pollen would greatly enhance tolerance of stresses such as drought and salinity.

8.3.2 Social impacts

An ACIAR-funded study in salt-affected areas of Pakistan and similar studies in drought-affected areas of eastern India have shown that the education of girls is one of the first casualties when farming families suffer a loss of income. The greater yield and yield stability of hybrid rice should enhance gender equity by providing girls with a more sustainable access to education.

8.3.3 Environmental impacts

The introduction of hybrid rice in China in the 1970s led to a 30% rise in rice production. This was due in part to the higher yield of hybrids compared with modern inbreds but also in part to the fact that hybrids were more effective than modern inbreds in replacing traditional low-yielding varieties. As a result, China was able to feed its rising population with a minimal increase in the area under cultivation. This saved many millions of hectares of forest and wetlands. In South and Southeast Asia, deforestation and loss of wetlands would have been much more marked without modern inbreds; the wider adoption of hybrids would enhance the protection of forested areas and wetlands.

8.4 Communication and dissemination activities

1. 5th International Rice Genetics Symposium (Manila, November 2005)

Drs. Abdul Chaudhury and John Bennett organized a well-attended workshop on “Reproductive Biology of Rice.” Project results were presented.

2. Phase 2 Mid-term Review Workshop (Canberra, August 2006).

This meeting was held at CSIRO Plant Industries and was attended by several staff from IRRI, CSIRO, and ACIAR, as well as the two external reviewers (Prof. Don Marshall and Dr. Enrico Perotti). Presentations were given by Drs. Bennett, Hervé, and Xie from IRRI and Drs. Luo and Chaudhury from CSIRO.

3. Gordon Research Conference (California, February 2007).

Dr. Bennett presented a report on IRRI's apomixis research.

4. Third International Symposium on Apomixis (Germany, July 2007).

The project was represented by Drs. John Bennett, Abdul Chaudhury, and Ming Luo and Ms. Xinai Zhao. Presentations were given by John Bennett and Ming Luo.

5. Terminal Review, IRRI (IRRI, Philippines, April 2008).

The meeting was attended by staff from IRRI and CSIRO, the external reviewer (Prof. Don Marshall), and three invited representatives of major rice-growing countries in Asia, who summarized their research related thematically or technically to apomixis research (Dr. Imran Siddiqi, scientist, Centre for Cellular and Molecular Biology, India; Dr. Inez Slamet-Loedin, Indonesian Institute of Sciences, Indonesia; Professor Yongzhong Xing,

Huazhong Agricultural University, China). Additional presentations were given by Dr. Bennett, Dr. Luo, and Dr. Anna Koltunow (Stream Leader, CSIRO Plant Industry).

6. International Epigenome Conference, Blue Mountain, Australia, September 2008.

Dr. Ming Luo presented a paper that included his research on rice Polycomb genes and their imprinting.

9 Conclusions and recommendations

This Phase 2 project, like the Phase 1 project before it, is unusual among ACIAR-funded projects in the degree to which it has required upstream research. That was recognized from the beginning by all parties, as indicated by the commitment in principle to three Phases over 15 years. The fact that the science emerging from the project has been consistently praised by the external reviewers is testament not only to the quality of the research but also to the potential impact of a successful outcome. This impact was quantified in an ex ante analysis by ACIAR itself (McMeniman and Lubulwa 1997). Here, we summarize the key lessons learned during Phase 2 and put forward a new vision for Phase 3 in the broader context of global apomixis research.

9.1 Conclusions

Objective 1 of Phase 2 was based on the proposal that the role of the FIS proteins of *Arabidopsis* in controlling the initiation of endosperm development would be conserved in their rice homologues. Our results establish clearly that this function has not been conserved in the rice FIS proteins, and at present is it unclear which rice proteins, if any, play this role. While it is true that the last common ancestor of rice and *Arabidopsis* existed some 200 million years ago, our proposal was not unreasonable, because conservation of both sequence and function between these species is probably more commonly observed than conservation of sequence with complete change/loss of function. Furthermore, as loss of function is impossible to predict, the proposal was well worth an experimental test.

The fact that the same CSIRO researchers who helped to discover *FIS* gene function in *Arabidopsis* have now shown convincingly that the same mechanism does not operate in rice will be a clear signal to researchers to put more effort into characterizing the control of endosperm formation in other species. It will be especially important to resolve this question for rice because several large benefits could accrue from including autonomous (fertilisation-independent) endospermy as a feature of apomictic rice. As this trait would eliminate the need for pollen, transgenic apomictic hybrid rice would find easier regulatory approval not only for the apomictic trait but also for most other transgenic traits included in the genotype. It would also greatly reduce the sensitivity of rice to abiotic stresses, which tend to reduce yield mainly through their impact on pollen development and function.

Under Objective 1, it was also planned to exploit fertilisation-independent formation of seed coat and pericarp, a phenomenon observed in several transgenic lines by both IRRI and CSIRO. Normally, the development of the seed coat and the pericarp (both maternal tissues) is halted at a poorly understood checkpoint until after fertilisation; by-passing this checkpoint would be an essential feature for apomictic hybrid rice devoid of pollen. However, both groups have found it difficult to separate the transgene-dependent occurrence of this phenomenon from a lower frequency occurrence in control plants. Intriguingly, the CSIRO scientists have preliminary evidence that in *Arabidopsis* the frequency of fertilisation-independent formation of seed coat and pericarp in control plants varies with genotype and environment. A similar effect in control rice plants would explain the results obtained by both IRRI and CSIRO. This lesson will be very important for Phase 3.

Objective 2 was based on the concept that aposporous initials (AIs) are derived from the same pool of cells that form extra sporocytes in the nucellus of the *mac1* mutant of maize. IRRI's goal was to reach three milestones: (i) to identify the rice homologue of *MAC1*, (ii) to use *OsMAC1* to induce extra megaspore mother cells (MeMCs) in the nucellus, and (iii) to convert these secondary MeMCs into AIs by eliminating meiosis. A knockout mutant of rice, *msp1*, was found in Japan to produce the same phenotype as the *mac1* mutant (Nonomura et al 2003). However, although there are now indications that MAC1 is not the

same protein as MSP1, its identity is still unknown (Ma et al 2007). The discovery by IRRI and CSIRO that OsTDL1A is a ligand of MSP1 (Zhao et al 2008) raises the possibility that OsTDL1A is OsMAC1, but it is also possible that a third protein, interacting with both MSP1 and OsTDL1A, has that property. A key lesson emerging from our work is that RNA interference (RNAi) targeted against OsTDL1A is preferable to a knockout mutant because the promoter that we used for RNAi (maize Ubiquitin1) causes the desired phenotype in the nucellus but fails to cause sterility in the anthers, unlike the *msp1* knockout mutant. Another lesson is that a careful comparison of the genomes of rice and budding yeast, the organism in which meiosis is best understood, can identify candidate genes for use in by-passing meiosis.

9.2 Recommendations

To ensure that this Phase 2 project ultimately has the desired impact for poor rice farmers in Asia and Africa, we recommend that (i) support be forthcoming for a revamped Phase 3 and that (ii) Phase 3 be integrated with new initiatives in global research on apomixis.

The objectives under Phase 3 will include the following:

(i) To identify a rice accession that shows little or no induction of seed coat and pericarp in the absence of fertilisation, probably as part of a collaboration with key laboratories expert in the regulation of rice seed formation.

(ii) To identify the protein complex that controls the initiation of autonomous endospermy in rice.

(iii) To convert secondary MeMCs into AIs by eliminating meiosis.

(iv) To initiate mitosis in AIs to form aposporous embryo sacs.

(v) To induce aposporous embryo sacs to form parthenogenic embryos.

(vi) To combine genetically outputs of (i), (ii), and (v) to produce synthetic apospory in rice.

Progress toward these objectives will be greatly aided by recent and emerging advances with natural aposporous apomicts such as *Hieracium piloselloides* (a dicot that was featured in Phase 1) and *Poa pratensis* and *Cenchrus* (both monocots and, like rice, members of the grass family), together with the initiation of molecular studies on *Boechera holboellii*, a diploid apomict closely related to the dicot *Arabidopsis*. Catanach et al (2006) used deletion mapping to identify genomic regions involved in apomeiosis and parthenogenesis in *Hieracium*. Sequencing of these regions is under way, aided by the mutants such as loss of apomeiosis1 (loa1) in *Hieracium* (Okada et al 2007). Matzke et al (2005) deduced that fully sexual and fully apomictic lines of *Poa pratensis* differ in five genetic loci, three concerned with apomeiosis and two with parthenogenesis. Conner et al (2008) sequenced bacterial artificial chromosome clones from the apospory-specific genomic region of *Pennisetum squamulatum* and *Cenchrus ciliaris*, identifying numerous candidate genes that may be required for apospory. Kantama et al (2007) established that diploid apomicts of the *Boechera holboellii* complex display large-scale chromosome substitutions and aberrant chromosomes compared with the diploid sexual species *B. stricta*. Most recently, and very encouraging, Ravi et al (2008) reported that the dyad mutant of *Arabidopsis* shows a low frequency of apomeiosis as a result of replacing the equational division of meiosis I by a division more akin to mitosis.

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10.2 List of publications produced by project

1. Bi X, Khush GS, Bennett J. 2005. The rice nucellin gene ortholog OsAsp1 encodes an active aspartic protease without a plant-specific insert and is strongly expressed in early embryo. Plant Cell Physiol. 46:87-98.
2. Gamuyao R. M.Sc. thesis, University of the Philippines at Los Baños, Philippines, April 2007. Expression analysis of WOX (Wuschel-related homeobox) genes in rice (*Oryza sativa* L.).
3. Zhao XA. Ph.D. thesis, Zhejiang University, Hangzhou, China, March 2008. Towards apospory in rice through the study of genes controlling the number of megaspore mother cells.
4. Zhao XA, de Palma J, Oane R, Gamuyao R, Luo M, Chaudhury A, Hervé P, Xue QZ, Bennett J. 2008. OsTDL1A binds to the LRR domain of rice receptor kinase MSP1 and is required to limit sporocyte numbers. Plant J. 54:375-387.
5. Bennett J, Zhao XA. 2008. No sex please: we're apomicts. Rice Today 7(3):34-35.
6. Luo M, Platten D, Chaudhury A, Peacock WJ, Dennis ES. Evolutionary relationship, expression and imprinting of rice homologs of the Polycomb Group genes *E(z)*, *Su(z)12*, and *ESC*. Submitted.
7. Zhou W. Ph.D. thesis, China Agricultural University, Beijing, China, pending. Gene expression in primary and secondary megaspore mother cells of rice.