OsTDL1A binds to the LRR domain of rice receptor kinase MSP1, and is required to limit sporocyte numbers

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Summary

Hybrids lose heterotic yield advantage when multiplied sexually via meiosis. A potential alternative breeding system for hybrids is apospory, where female gametes develop without meiosis. Common among grasses, apospory begins in the nucellus, where aposporous initials (AIs) appear near the sexual megaspore mother cell (MeMC). The cellular origin of AIs is obscure, but one possibility, suggested by the mac1 and msp1 mutants of maize and rice, is that AIs are apomeiotic derivatives of the additional MeMCs that appear when genetic control over sporocyte numbers is relaxed. MULTIPLE SPOROCYTES1 (MSP1) encodes a leucine-rich-repeat receptor kinase, which is orthologous to EXS/EMS1 in Arabidopsis. Like mac1 and msp1, exs/ems1 mutants produce extra sporocytes in the anther instead of a tapetum, causing male sterility. This phenotype is copied in mutants of TAPETUM DETERMINANT1 (TPD1), which encodes a small protein hypothesized to be an extracellular ligand of EXS/EMS1. Here we show that rice contains two TPD1-like genes, OsTDL1A and OsTDL1B. Both are co-expressed with MSP1 in anthers during meiosis, but only OsTDL1A and MSP1 are co-expressed in the ovule. OsTDL1A binds to the leucine-rich-repeat domain of MSP1 in yeast two-hybrid assays and bimolecular fluorescence complementation in onion cells; OsTDL1B lacks this capacity. When driven by the maize Ubiquitin1 promoter, RNA interference against OsTDL1A phenocopies msp1 in the ovule but not in the anther. Thus, RNAi produces multiple MeMCs without causing male sterility. We conclude that OsTDL1A binds MSP1 in order to limit sporocyte numbers. OsTDL1A-RNAi lines may be suitable starting points for achieving synthetic apospory in rice.

Keywords: meiosis, apospory, leucine-rich-repeat receptor kinase, RNA interference, rice.

Introduction

Hybrids out-perform inbreds under favorable and unfavorable conditions, but they lose heterotic yield advantage when multiplied sexually because of segregation and recombination occurring during meiosis (Bi et al., 2003). A possible alternative breeding system for hybrids is apospory, a form of seed production in which the embryo arises from a nucellar cell without meiosis (Bicknell and Koltunow, 2004). Apospory is common among grasses, including relatives of several major cereal crops (Carman, 1997), but it is unknown in rice and its relatives (Brar et al., 1995). We have initiated a project to develop synthetic apospory for hybrid rice (Bi et al., 2003).

Naturally occurring apospory begins with the appearance of aposporous initials (AIs) alongside the megaspore mother cell (MeMC) of the ovule (Bicknell and Koltunow, 2004). Like the MeMC, AIs are enlarged nucellar cells that have the capacity to develop into embryo sacs, but because they do
so without undergoing meiosis, the resulting embryo sacs are diploid rather than haploid. Diploid egg cells can develop into embryos by parthenogenesis, i.e. without fertilization, producing progeny that are genetically identical to the maternal tissue (Matzk et al., 2005).

The cellular origin of AIs is uncertain, but our hypothesis is that AIs develop from the same group of nucellar cells that form extra MeMCs when a genetic control limiting MeMC numbers is relaxed. The existence of this control was first suggested by studies on the *multiple archaesporeal cells* (*mac1*) mutant of maize, which produced extra sporocytes in both ovule (Sheridan et al., 1996) and anther (Sheridan et al., 1999). A similar set of phenotypes was seen in the *multiple sporocytes* (*msp1*) mutant of rice (Nonomura et al., 2003). As *msp1* was a Tos17 insertion mutant, the insertion site could be cloned and thus identified as a member of the leucine-rich-repeat (LRR) receptor kinase gene family.

MSP1 is closely related structurally and functionally to EXS/EMS1 of Arabidopsis. Both *exs* and *ems1* mutants produce extra sporocytes in the anther (Canales et al., 2002; Zhao et al., 2002). Another Arabidopsis mutant, *tapetum determinant1* (*tpd1*), phenocopied the *exs/ems1* mutants, but whereas EXS/EMS1 encoded an LRR receptor kinase, *TPD1* encoded a small, putatively extracellular protein (Yang et al., 2003, 2005). Ma (2005) hypothesized that *TPD1* is a ligand for the extracellular LRR domain of EXS/EMS1. Here, we establish that rice contains two close homologues of *TPD1* (*OsTDL1A* and *OsTDL1B*). We examine the sites of expression of these genes relative to *MSP1*, examine the affinity of *OsTDL1A* and *OsTDL1B* proteins for the LRR domain of MSP1, and study the effect of RNA interference of *OsTDL1A* on anther and ovule. We confirm that MSP1 and its close paralog MSP1-like1 (MSL1) are structurally the most similar rice proteins to EXS/EMS1.

**Results**

*OsTDL1A* and *OsTDL1B* are rice homologs of *TPD1* of Arabidopsis

The full-length protein sequence of Arabidopsis TPD1 ([AAR25553, 176 amino acids (aa)]) was used as the query in a TBLASTN search of the rice genome. We detected two TPD1-like genes and named them *OsTDL1A* (BLAST e-value: \(5 \times 10^{-27}\)) and *OsTDL1B* (BLAST e-value: \(1 \times 10^{-18}\)). They are located on chromosomes 12 and 10, respectively, and the corresponding full-length cDNAs are AK108523 and AK121594. When their predicted protein sequences (NP_001066753, 226 aa; NP_001064316, 169 aa) were used as TBLASTN queries of the Arabidopsis genome, the best hits were to TPD1 and another Arabidopsis protein (ABF59206, 179 aa), which we have named AtTDL1. TPD1 is more similar to AtTDL1 (e-value: \(2 \times 10^{-38}\)) than *OsTDL1A* is to *OsTDL1B* (e-value: \(2 \times 10^{-14}\), a conclusion supported by CLUSTALW analysis (Figure 1A). The four proteins cluster separately from the next most similar proteins encoded by the rice and Arabidopsis genomes (the boxed proteins are from rice).

The greater length of *OsTDL1* compared with AtTDL1, *OsTDL1A* and *OsTDL1B* results principally from four inserts of about 17, 12, 6 and 6 aa in length (Figure 1b). The amino acid identities between TPD1 and any of the other three proteins are highlighted, and are located mainly in the C-terminal half. SignalP-NN software (Nielsen et al., 1997) predicts that all four proteins contain cleavable N-terminal signal peptides (underlined) to target them into the endoplasmic reticulum for export from the cell. Mature molecules of *OsTDL1A* and *OsTDL1B* are predicted to contain 191 and 154 aa, respectively.
Expression of OsTDL1A, OsTDL1B and MSP1 in rice organs

OsTDL1A and OsTDL1B were compared with MSP1 in terms of spatial and temporal regulation of gene expression (Figure 2). RNA was extracted for RT-PCR from roots, young shoots, flag leaves and spikelets of various stages of development. Before flowering the spikelet stages were defined in terms of spikelet length (1, 3 and 7 mm), with meiosis occurring in anthers and ovules mainly at 3 mm; spikelet samples were also taken at flowering (0 days after flowering (DAF)) and five days later (5 DAF). For each gene, RT-PCR primer sequences were located in exons flanking introns (Figure S1). Single amplicons of the expected size for OsTDL1A and OsTDL1B (546 and 485 bp, respectively) were generated from RNA of roots and spikelets (1 mm, 3 mm and 5 DAF). Three amplicon sizes were seen for MSP1, as was also reported by Nonomura et al. (2003). The sizes of these amplicons are consistent with the sizes expected for the fully spliced transcript (507 bp), a transcript in which only the first intron has been removed by splicing (752 bp) and an unspliced transcript (1298 bp), based on the full-length cDNA sequence (AK120933). The smallest amplicon was amplified most strongly from 1- and 3-mm spikelets. The largest amplicon was not a PCR product derived from possible DNA contamination, because the RNA preparations lacked DNA contamination as judged by the failure of the GAPDH primers to produce the genomic amplicon (Figure 2, open arrow). We conclude that MSP1, OsTDL1A and OsTDL1B are all expressed in spikelets before and during meiosis.

Unlike MSP1, OsTDL1A and OsTDL1B were also expressed in roots. Yang et al. (2003) found that TPD1 is expressed in the leaves and young seedlings of Arabidopsis, whereas EXS/EMS1 is not expressed in those tissues; roots were not examined. It is not clear why TPD1 and its rice homologs OsTDL1A and OsTDL1B are expressed in tissues where transcripts of the receptor kinases are absent. One possibility is that they also interact with proteins other than EXS/EMS1 and MSP1 to regulate processes other than entry into meiosis. A close paralog of MSP1 (MSL1, see Figure S1) was expressed in all tissues examined, including the root (Figure 2), and might interact with OsTDL1A or OsTDL1B. To our knowledge, the expression pattern of AtTDL1 (Figure 1) has not yet been reported.

Localization of MSP1, OsTDL1A and OsTDL1B transcripts within spikelets

RNA in situ hybridization on 3-mm spikelets indicated that MSP1, OsTDL1A and OsTDL1B were expressed most strongly in the anther, and more weakly in the lemma, palea and basal tissue of the spikelet; transcripts of MSP1 and OsTDL1A were also detected in the ovule, but transcripts of OsTDL1B were below the limit of detection. Figure 3 focuses on the expression patterns of the three genes in the ovule and anthers of wild-type cv. Nipponbare and the homozygous msp1 mutant. All three transcripts were detected with antisense probes (Figure 3), but essentially no transcripts were detected with sense probes (only shown in Figure 3 for OsTDL1A). The dotted lines in Figure 3 show the outlines the nucellus, with one or more MeMCs inside (arrowed) and the integuments outside. In wild-type plants, transcripts of MSP1 and OsTDL1A, but not OsTDL1B, were clearly seen throughout the ovule (nucellus and integuments), except for the single MeMC. In the homozygous msp1 mutant, transcripts of both MSP1 and OsTDL1B were difficult to detect in the ovule, but transcripts of OsTDL1A were clearly seen throughout the ovule, except for the multiple MeMCs.

The results for MSP1 agree with those obtained by Nonomura et al. (2003), but the results for OsTDL1A and OsTDL1B are new, and suggest that (i) OsTDL1A is much more abundantly expressed in the ovule than OsTDL1B, (ii) OsTDL1A and MSP1 tend to be co-expressed throughout the non-sporogenous cells of the ovule, and (iii) neither gene is expressed significantly in the MeMCs. We cannot compare these results with data on EXS/EMS1 or TPD1 because RNA in situ hybridization data for these genes have not yet been reported for the Arabidopsis ovule.

MSP1, OsTDL1A and OsTDL1B were clearly expressed in the anther wall of wild-type plants, but were not expressed in microspore mother cells (MiMCs). In the msp1 mutant, OsTDL1A was strongly expressed in the anther wall, in spite of the absence of the tapetum and other layers (Nonomura et al., 2003). This agrees with data from Arabidopsis, where TPD1 is expressed in the anther wall in the wild type and in the ems1 mutant (Yang et al., 2003). The implication is that in rice and Arabidopsis, OsTDL1A and TPD1 are expressed in certain anther wall cells irrespective of whether they differentiate into a tapetum. The difference between our data and
those from Arabidopsis (Ma, 2005; Yang et al., 2003) lies in our failure to detect transcription of OsTDL1A or OsTDL1B in the MiMCs. There is no evidence that OsTDL1A resembles TPD1 in becoming increasingly expressed in MiMCs as the anther matures (Ma, 2005).

We conclude that, in both ovule and anther, OsTDL1A and MSP1 are expressed in similar cell types. In the ovule both genes are widely expressed, but not in the MeMC. In the anther, both genes are expressed in the anther wall. OsTDL1B is expressed in the anther wall but not in the ovule.

**Evidence for physical interaction between OsTDL1A and the LRR domain of MSP1**

MSP1 is predicted by SignalP-NN, InterProScan and PSORT to be an LRR receptor kinase residing in the plasma membrane, with its LRR domain on the outside of the membrane and its serine/threonine kinase domain in the cytosol. The N-terminal signal peptide would lead MSP1 co-translationally into the endoplasmic reticulum, but the protein would be retained within the plasma membrane by virtue of a membrane-spanning domain located between amino acids I919 and A934. To examine whether OsTDL1A or OsTDL1B have the potential to interact physically with the LRR domain of MSP1, we employed two-hybrid analysis in yeast cells and bimolecular complementation in onion cells.

**Yeast two-hybrid analysis.** The yeast strain used for two-hybrid analysis requires leucine, tryptophan and histidine for growth, but will grow in their absence if a modified HIS3 gene is activated by co-transformation of the strain with two plasmids that will be maintained by their ability to satisfy the leucine and tryptophan requirements, respectively (James et al., 1996). In this strain, the HIS3 coding region is under the control of the ADH1 promoter, which binds the GAL4 transcription factor, either as an intact protein or as a complex of its DNA-binding domain and its activation domain. We expressed the DNA-binding domain as a fusion protein with MSP1Δ, a truncated form of MSP1. MSP1Δ contains the first 894 aa of MSP1, including a 34-unit LRR domain, but it lacks the transmembrane domain and the tyrosine kinase domain. Such fusion proteins are directed to the nucleus by the nuclear-targeting signal on the DNA-binding domain. The plasmid carrying the fusion gene provides another gene that satisfies the leucine requirement. The activation domain of HIS3 was also expressed as a fusion protein with the entire protein molecule of either OsTDL1A or OsTDL1B. This fusion protein was directed to the nucleus by a nuclear-targeting signal on the activation domain, and the plasmid carrying it also satisfied the tryptophan requirement. Physical interaction between MSP1Δ and OsTDL1A or OsTDL1B inside the nucleus of yeast cells would result in yeast growth on medium deficient in leucine, tryptophan and histidine. Transformed yeast grew on this medium when both MSP1Δ and OsTDL1A or OsTDL1B were co-expressed in the cells, but did not grow when either construct was omitted or when OsTDL1A was replaced by OsTDL1B (Figure 4A).

**Bimolecular fluorescence complementation in onion cells.** This method depends on the reconstitution of an active yellow fluorescent protein (YFP) from two separated domains (Bracha-Drori et al., 2004). The N-terminal domain (YN) was fused with MSP1Δ, and the C-terminal domain (YC)
was fused with OsTDL1A or OsTDL1B. Co-expression of the MSP1Δ and OsTDL1A fusions in onion epidermal cells reconstituted the expected green fluorescence, but co-expression of MSP1Δ and OsTDL1B failed to do so (Figure 4b).

We conclude that the two methods used for Figure 4 indicate that OsTDL1A has the capacity to interact physically with the LRR domain of MSP1. Neither method suggested that OsTDL1B has this capacity, at least under the assay conditions in yeast and onion cells. This is consistent with a marked difference in protein sequence between OsTDL1A and OsTDL1B in the region of the protein most highly conserved with TPD1 and AtTDL1 (indicated in Figure 1b).

**RNA interference suppresses OsTDL1A transcript levels in spikelets**

The proposal that TPD1 might interact physically with EXS/EMS1 (Ma, 2005) was based on genetic evidence that the tcp1 mutant phenocopies the exs and ems1 mutants in blocking tapetum development and pollen formation in Arabidopsis (Canales et al., 2002; Yang et al., 2003, 2005; Zhao et al., 2002). To check whether downregulation of OsTDL1A transcription might phenocopy the tcp1 mutant, we produced RNAi lines in which the OsTDL1A gene was targeted. Primary embryogenic calli were co-cultivated with Agrobacterium harboring the pANDA vector, in which an OsTDL1A-RNAi cassette was under the control of the maize ubiquitin1 promoter. Transformants were selected and regenerated on hygromycin. Of the 50 independent T0 transformants, 47 were positive for the HPT gene, and 46 of the HPT+ plants were also positive for the GUS marker located within the RNAi cassette (Miki and Shimamoto, 2004). All HPT-GUS+ transformants were positive for expression of the RNAi cassette, as judged by RT-PCR of the GUS marker. Figure 5(a) shows PCR amplicons from the HPT gene and the GUS fragment in seven regenerants, and their absence in a non-transgenic control. All regenerants expressed the RNAi cassette as judged by RT-PCR of the GUS fragment. Furthermore, these seven plants showed a high degree of suppression of OsTDL1A transcripts. OsTDL1B and MSP1 transcripts were not downregulated in these plants (Figure 5a).

**RNAi of OsTDL1A phenocopies the msp1 mutant in the ovule, but not in the anther**

Morphological and histological analyses were conducted on nine independent T0 OsTDL1A-RNAi plants to determine whether they phenocopied the homozygous msp1 mutant in anther and ovule. The most conspicuous phenotypes of the msp1 mutant are reduced anther size and complete male sterility (Nonomura et al., 2003), but neither of these phenotypes was seen with OsTDL1A-RNAi plants, which showed normal anther size and good fertility for greenhouse conditions. Light microscopy confirmed the lack of msp1 phenotypes in the anthers of OsTDL1A-RNAi plants. Figure 6 presents data obtained with one of the T0 OsTDL1A-RNAi plants (#4363). As in the wild-type, the tapetum was present in #4363 (Figure 6a), and large quantities of callose were associated with the tetrads (Figure 6b). Anthers of msp1 plants lacked the tapetum, and the locule was packed with MiMCs lacking callose.

By contrast, seven T0 OsTDL1A-RNAi plants phenocopied the msp1 mutant in the ovule and produced multiple MeMCs. In the case of #4363, the presence of multiple MeMCs (Figure 6c) was accompanied by reduced callose accumulation (Figure 6d), as also seen for the msp1 mutant. We examined whether the multiple MeMCs were capable of undergoing meiosis by staining for leptotene–pachytene–zygotene figures (Figure 6e). Wild-type ovules contained no more than a single MeMC undergoing meiosis, whereas ovules of OsTDL1A-RNAi and msp1 plants contained several such cells. To estimate the number of multiple MeMCs per ovule, and the fraction of them at pachytene, it was necessary to examine serial sections. OsTDL1A-RNAi line #4363 contained about 10 MeMCs per ovule, whereas msp1 ovules contained about 15 MeMCs. The fraction of MeMCs that were at leptotene–pachytene–zygotene in any given set of serial sections was about 30%. It is not clear whether this percentage reflects a lack of synchrony among putative MeMCs in undergoing meiosis,
or a failure of many MeMCs to reach leptotene–pachytene–zygotene stages. Sheridan et al. (1996) attributed a low percentage of maize MeMCs undergoing meiosis in such sections to asynchrony.

Although the ovule of the msp1 mutant contains multiple MeMCs, only one distinct embryo sac is usually observed (Nonomura et al., 2003). However, this embryo sac is surrounded by additional cell-like structures that are absent from wild-type ovules (Figure 6f). These additional structures are also adjacent to the embryo sac of OsTDL1A-RNAi ovules (Figure 6f).

OsTDL1A-RNAi remains effective in the ovule of the T1 generation

T1 plants derived from T0 plant #4363 were examined by PCR for the presence of the construct (HPT gene) and the RNAi cassette (GUS marker). They were also examined by RT-PCR for expression of the cassette (GUS marker) and its effectiveness (downregulation of OsTDL1A). Figure 5(b) shows the analysis of three HPT’GUS’ T1 plants and one HPT’GUS’ T1 plant, together with a HPT’GUS’ non-transgenic control. When RT-PCR was conducted on RNA
extracted from 3-mm spikelets of the five plants, only the HPT"GUS" T$_1$ plants expressed the GUS marker and downregulated the OsTDL1A gene. Furthermore, only these plants exhibited multiple MeMCs in the ovule. However, all five plants were male-fertile as judged by the normal appearance of the anthers and the production of seeds. We conclude that the OsTDL1A-RNAi construct had been transmitted to, and had segregated within, the T$_1$ generation, where it was expressed and effective in downregulating OsTDL1A transcript levels in spikelets and inducing multiple MeMCs in the ovule without affecting the anther.
Examination of the failure of OsTDL1A-RNAi to phenocopy msp1 mutants in the anther

We hypothesized that the failure of OsTDL1A-RNAi to phenocopy msp1 in the anther could be caused by (i) a difference between ovule and anther in the control of meiocyte numbers, or (ii) the failure of RNAi to downregulate OsTDL1A in the anther. The second explanation is suggested by the report that the maize ubiquitin1 promoter, used here to drive expression of the RNAi cassette, is not normally expressed in the rice tapetum (Cornejo et al., 1993), which is however a major site of OsTDL1A expression. RNA in situ hybridization (Figure S2) showed that the tapetum of T2 florets in OsTDL1A-RNAi line #4363 yielded a hybridization signal with the antisense probe that was substantially higher than the signal obtained with the sense probe. By contrast, in the ovule, RNAi resulted in an antisense hybridization signal similar to that observed with the sense probe, and considerably lower than the antisense signal in the ovule of non-transformed plants. However, examination of three replicate slides for both RNAi plants and control plants, including the slides shown in Figure S2, indicated that the antisense signal for anthers was consistently higher in controls than in RNAi plants. These results suggest that RNAi does reduce the OsTDL1A transcript level in the anthers, but not to the extent required to produce a phenotype.

As the maize ubiquitin1 promoter contains cis-elements responsive to heat shock (Streatfield et al., 2004), we explored the possibility that a controlled heat shock might lead OsTDL1A-RNAi lines to phenocopy msp1 in the anther by increasing the degree of downregulation of the RNAi cassette. We measured the effect of a heat shock at the booting stage on spikelet fertility (Figure S3a) and assayed expression of the RNAi cassette by RT-PCR of the GUS marker in flag leaves (Figure S3a). We also examined the expression of two representatives of the class-I small heat shock protein genes of rice (Guan et al., 2004). It is evident that 1 h of heat shock at 45°C greatly reduced the fertility of the OsTDL1A-RNAi line, whereas it only produced a minor reduction in fertility of the non-transformed control. A heat shock at 40°C (for 0.5 or 3 h) produced a much smaller reduction in fertility in both RNAi plants and non-transgenic controls. Expression of the RNAi cassette (GUS marker) in flag leaves was detectable in unstressed transgenic plants, and was upregulated approximately equally by the two intensities of heat shock; expression was absent in stressed and unstressed non-transgenic plants. By contrast, two endogenous heat-shock genes studied previously by Guan et al. (2004) were markedly more responsive at 45°C than at 40°C in both RNAi plants and controls, as indicated by the requirement for only 20 cycles of RT-PCR compared with 30 cycles for the GUS marker. These results suggest that severe heat shock can cause selective sterility in OsTDL1A-RNAi line #4363 by a mechanism requiring interaction between the RNAi cassette and the endogenous heat-shock response.

Discussion

OsTDL1A binds to the LRR domain of MSP1

Our data establish that rice contains two close homologs of TPD1, namely, OsTDL1A and OsTDL1B. Only the more similar homolog, OsTDL1A, appears to have a capacity to bind to the LRR domain of MSP1, as judged by two-hybrid analysis in yeast cells and bimolecular complementation in onion cells. OsTDL1B shows no evidence of having a physical affinity for the LRR domain of MSP1. As the LRR domain is extracellular, it is appropriate that the SignalP-NN algorithm predicts that OsTDL1A is secreted from the cell, where it would be able to interact with the LRR domain. It would be intriguing to transform rice with a construct encoding OsTDL1A or OsTDL1B modified with a C-terminal tag (e.g. hexahistidine) to identify their protein partners. This sort of in vivo experiment would presumably confirm that OsTDL1A associates with MSP1, and would also throw light on the roles of OsTDL1A and OsTDL1B in tissues such as roots, where MSP1 is not expressed. It may also reveal other proteins associated with MSP1 and OsTDL1A.

The validity of predictions of protein–protein interactions based on yeast two-hybrid analysis was evaluated by Deane et al. (2002). They presented four conditions that enhance confidence in the predicted interactions: (i) known functional relationship between the two proteins, (ii) co-expression of the two genes, (iii) existence of evidence for interactions between paralogs of the two proteins, and (iv) agreement between yeast two-hybrid data and data obtained by another method. Our study of OsTDL1A and MSP1 satisfies conditions (i), (ii), and (iv), and may in future satisfy condition (iii). Criterion (i) is satisfied by the fact that OsTDL1A-RNAi phenocopies the msp1 mutant in the ovule. Criterion (ii) is satisfied by the co-expression of the two genes in the ovule and the anther. Criterion (iv) is satisfied by the agreement between yeast two-hybrid data and bimolecular fluorescence complementation data. Future work will examine whether OsTDL1B, a paralog of OsTDL1A, interacts with MSL1, a paralog of MSP1.

OsTDL1A-RNAi phenocopies the msp1 mutant in the ovule, but not in the anther

OsTDL1A-RNAi, when driven by the ZmUbiquitin1 promoter, downregulates OsTDL1A transcript levels without affecting transcript levels for OsTDL1B or MSP1. These transgenic events copy the phenotype of the msp1 mutant in the ovule by producing multiple MeMCs and developing an embryo...
We find that OsTDL1A expression and possible roles of TPD1/OsTDL1A sites of expression and possible roles of TPD1/OsTDL1A difficult to detect the phenotype in the ovule of Arabidopsis. MeMCs that can form. If that were the case, it may be more with the marked induction of two endogenous heat-shock proteins presumably forming a complex on the outside surface of the cells, but this remains to be established. In the ovule, the zone of overlap between OsTDL1A expression and MSP1 expression is much larger than the zone of cells capable of forming extra MeMCs. This suggests that at least one additional component is required to define the cells that will be induced to become MeMCs upon inactivation of the MSP1-OsTDL1A receptor complex. The weak expression of MSP1 and OsTDL1A in tissues such as the palea and lemma may have no functional consequence if these additional component(s) are not co-expressed in those tissues. Consideration of our data and those of Sheridan et al. (1996) for the ma1 mutant of maize suggests that the additional component(s) may convey positional information defining the L2 layer. This hypothetical component could be extracellular, plasma membrane-bound or cytosolic. The brassinosteroid receptor BRI1 is a close relative of EXS/EMS1/MSP1 (Figure S1), and is known to interact with extracellular brassinosteroid through its LRR domain (He et al., 2000). Within the plasma membrane it activates through homodimerization (Wang et al., 2005), and association with BAK1, a second LRR receptor kinase (Russinova et al., 2004). It is negatively regulated by BK1, a cytosolic protein (Wang and Chory, 2006). Further research on the MSP1/OsTDL1A receptor complex may reveal comparable features. The fact that BRI1 is located in endomembranes as well as the plasma membrane (Geldner et al., 2007) indicates that importance should be given to determining the subcellular location of MSP1 and OsTDL1A, and we should entertain the possibility that OsTDL1A is cleaved to an active form, like CLAVATA3, the ligand of another LRR receptor kinase, CLAVATA1 (Ni and Clark, 2006). It will be particularly interesting to see the identification of the MAC1 gene, which appears not to be the maize ortholog of MSP1 (Ma et al., 2007). It could be the maize ortholog of OsTDL1A, or it could correspond to yet another component of the ligand-receptor system.

Both MSP1 (Nonomura et al., 2003) and OsTDL1A (X. Zhao, unpublished data) are expressed throughout the nucellus prior to meiosis, whereas the primary MeMC

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appears not to express these genes, and the extra MeMCs do not appear to express OsTDL1A. This result suggests that entry into meiosis and termination of MSP1 and OsTDL1A expression are somehow connected. One possibility is that the termination of MSP1 and OsTDL1A expression is a consequence of the entry into meiosis; another is that termination of expression is part of the signal leading to meiosis. The second possibility is consistent with the induction of meiosis in a limited number of nucellar cells of the L2 layer by the mac1 and msp1 mutations, and OsTDL1A-RNAi. A crucial question is the role of the L1 layer in this induction, comparable with the role of the L1 layer in the shoot meristem (Kessler et al., 2006; Lenhard and Laux, 2003).

Roles of OsTDL1B and AtTDL1

Our data suggest that OsTDL1B does not bind to the LRR domain of MSP1. We may learn more about the function of OsTDL1B from the analysis of OsTDL1B-RNAi plants that are currently being developed. One possibility is that OsTDL1B binds to MSL1, a rice paralog of MSP1. The MSL1 gene is located on chromosome 2, but appears to have been incorrectly annotated in the public databases; we offer a new annotation in Figure S1b, supported by partial sequencing of MSL1 cDNA (Figure S1c). Unlike rice, Arabidopsis appears to have no close paralog of EXS/EMS1 (Figure S1d). However, we reported in Figure 1 that Arabidopsis encodes a close paralog of TPD1, designated here as AtTDL1. It will be interesting to determine whether both TPD1 and AtTDL1 bind to the LRR domain of EXS/EMS1.

Implications of fertile OsTDL1A-RNAi lines for achieving synthetic apomixis in rice

We hypothesized that AIs originate from the same nucellar cells that form extra MeMCs when the control mechanism limiting the number of MeMCs in the ovule is relaxed. In rice, that relaxation may be produced by interfering with the function of MSP1 (Nonomura et al., 2003) or OsTDL1A (this report). However, at least one additional behavioral change is required to produce AIs: the extra MeMCs must bypass meiosis and enter directly into diploid mitosis to produce aposporous embryo sacs. We suggest that OsTDL1A-RNAi lines are superior to the msp1 mutant as a platform for producing this behavioral change because of their fertility, which is manifested in both the T0 and T1 generations. Although our results indicate that the OsTDL1A-RNAi line #4363 is more susceptible to severe heat shock (45°C) than the non-transformed control, it is unlikely that the temperatures normally encountered in the field will jeopardize the use of the RNAi approach for achieving apomixis in hybrid rice.

Our approach to synthetic apomixis has been influenced by studies on Kentucky Bluegrass (Poa pratensis), where sexual and apomictic lines differ at five genetic loci (Matzk et al., 2005). These loci are termed apomixis prevention (APV, apv), apomixis initiation (AIT, ait), megasporogenesis prevention (MDV, mdv), parthenogenesis prevention (PPV, ppv) and parthenogenesis initiation (PIT, pit), where the lower case allele is recessive. Full sexuality corresponds to one allele set (APV, ait, MDV, PPV, PIT), whereas full apomixis corresponds to another (apv, AIT, mdv, ppv, PIT). Matzk et al. (2005) suggested that APV may correspond to PpMAC1. Our results and those of Nonomura et al. (2003) suggest that APV may be PpMSP1 or PpTDL1A, with apv being a mutant allele relaxing control over MeMC numbers. We are currently examining candidate genes to play the role(s) of AIT and mdv.

Experimental procedures

Plant materials

Seeds of rice (Oryza sativa L. cv. Nipponbare, IRTP number 06669) were obtained from the International Network for the Genetic Evaluation of Rice at the International Rice Research Institute (IRRI, http://www.irri.org/). Plants were grown individually in pots of soil under glasshouse conditions with natural lighting and fertilization as described by Ji et al. (2007). For RNA extraction, tissue samples were frozen immediately in liquid nitrogen and stored temporarily at –80°C. The samples included roots and shoots from 10 days after germination (DAG), leaves harvested at 30 DAG, and spikelets harvested at 1, 3 or 7 mm in length (before flowering) and at 0 and 5 DAF.

Bioinformatics

MSP1, EXS/EMS1 and TPDP1 protein sequences were obtained from the NCBI database (http://www.ncbi.nlm.nih.gov), and were used in tBLASTn searches (http://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://www.arabidopsis.org and http://www.irri.org/). Plants were grown individually in pots of soil under glasshouse conditions with natural lighting and fertilization as described by Ji et al. (2007). For RNA extraction, tissue samples were frozen immediately in liquid nitrogen and stored temporarily at –80°C. The samples included roots and shoots from 10 days after germination (DAG), leaves harvested at 30 DAG, and spikelets harvested at 1, 3 or 7 mm in length (before flowering) and at 0 and 5 DAF.

Microscopy

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 stained in fast green and safranin or aniline blue.
Sections were viewed under a bright-field microscope (Carl Zeiss, http://www.zeiss.com), supported by IMAGE-PRO PLUS 5.1 software (Media Cybernetics, http://www.mediacy.com).

RNA extraction and RT-PCR

Total RNA was extracted from different tissues by Trizol, according to the instructions from the manufacturer (Invitrogen, http://www.invitrogen.com). RNA was quantified as described by Ji et al. (2005). RNA samples were then treated with RNase-free DNase (Promega, http://www.promega.com/) to remove any contaminating genomic DNA. Gene-specific amplification was conducted on cDNAs corresponding to transcripts of MSP1, MSL1, OsTDL1A, OsTDL1B, a segment of the Escherichia coli β-glucuronidase gene (GUS) and two endogenous rice heat-shock genes, Oshsp16.9A and Oshsp17.9A, described by Guan et al. (2004). The primer pairs used in reverse transcription-polymerase chain reaction (RT-PCR) were as follows (forward reverse, written from 5’ to 3’): MSP1 (ATCTCCACGTTTTATAGCTT-TAC, CTAGCGAGAGTAAAGCAGC); MSL1 (CAT-ACTAGCGGAGTCCCA, GACTGCTAGGATCTACAG); OsTDL1A (AACCTCTACTACTCTCCT, TCATACGACCCAGGTGTA); OsTDL1B (AGGTCTGAGGATCTACAG); GUS (CATGAGATGGGACACCATTGACTCATG); Oshsp16.9A (GCCTCTAGATGACATCCTGG, CCTCAACGAG-AAGACTCTAA); Oshsp17.9A (GCTATGCGGTGCGTTA, CTGACACGAGGCGACACAGTTAGT).

The locations of the primers within the rice genomic sequence are given in Figure S1(a). In each case the primers flanked introns to permit a clear distinction to be made between expected RT-PCR and PCR products, and to identify incomplete splicing. RT-PCR for each primer was performed (forward, reverse, written from 5’ to 3’): OsTDL1A (CCCTACTACACTCTCCTC, TTCTCT-TGGCAGGATTGACG); OsTDL1B (AGGTCTGAGGATCTACAG); GUS (CATGAGATGGGACACCATTGACTCATG); Oshsp16.9A (GCCTCTAGATGACATCCTGG, CCTCAACGAG-AAGACTCTAA); Oshsp17.9A (GCTATGCGGTGCGTTA, CTGACACGAGGCGACACAGTTAGT).

The method of Bracha-Dori et al. (2004) was used. The entry clones pDONTR™ 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, GGGGACAAGTTTGTACACAAAGCAGGCTTGGTACAAGAAAGCTGGGTATCCTG; OsTDL1B attB2, GGGGACCACTTTGTACAAAGAAGCTGGTTGCTACTG; OsTDL1A attB1, GGGGACAAGTTTGTACACAAAGCAGGCTTGGTACAAGAAAGCTGGGTATCCTG; OsTDL1B attB2, GGGGACCACTTTGTACAAAGAAGCTGGTTGCTACTG.

Yeast two-hybrid analysis. 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 attl sites were used to generate the hybrid containing the GAL4 AD (amino acids 768–881) and GAL4 DNA-BD (amino acids 1–147), respectively. Target sequences of OsTDL1A and OsTDL1B were recombined into the pACT2 vector, and the MSP1 LRR region was recombined into the pAS2 vector, by the LR reaction. Then, pACT2TDL1A and pACT2TDL1B were transformed into PJ69-4A yeast host strain using the lithium acetate procedure (James et al., 1996). A new introduction was verified by growing yeast on synthetic drop-out medium plates lacking 3-leucine. Using the same method, pAS2MSP1 and pACT2TDL1A or pACT2TDL1B were co-transformed into the yeast cells and selected on double-selective medium without 3-leucine and 3-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.

Bimolecular fluorescence complementation. The method of Bracha-Dori et al. (2004) was used. The entry clones pDONTR™
RNA interference of rice OsTDL1A gene

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: forward, from 5' to 3': CACCCTACCGTGGACGTTGA; GAGTTGACACGGGAGTGT. 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 E. coli DH5α cells and recovered from kanamycin-resistant colonies. The correctness of the cassette construction was verified by double digestion using Saci and Kpnl, which cut unique restriction enzyme sites in the pANDA vector. Transgenic rice (O. 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 the IRRI’s Confinement Level CL4 Transgenic Greenhouse under natural lighting conditions. Transformation was confirmed by PCR of the hygromycin phosphotransferase 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 downregulating 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.

Heat shock

Nipponbare (non-transgenic control) and OsTDL1A-RNAi plants at the booting stage were exposed to heat shock at 40°C for 0.5 or 3 h, and at 45°C for 10 min or 1 h in a Biotron Growth Cabinet, Model LPH-200-RD (NK Systems; Nippon Medical and Chemical Instruments; http://www.nihonika.co.jp). Humidity was set at 70%. Flag leaves were harvested 0.5 or 3 h after each treatment, and RNA was extracted and RT-PCR was conducted on samples. At grain maturity spikelet fertility was recorded.

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Supplementary Material

The following supplementary material is available for this article online:

Appendix S1. Annotation of MSL1.

Figure S1. Exon-intron structure of OsTDL1A, OsTDL1B and MSP1 and partial structure of MSL1.

Figure S2. Use of RNA in situ hybridization to detect OsTDL1A transcripts in the ovule and anther of 3 mm spikelets (stage of maximum meiosis) of non-transgenic plants (control) and T₀ plants of OsTDL1A-RNAi line #4363.

Figure S3. Effect of heat shock (HS) at the booting stage on spikelet fertility and gene expression in cv Nipponbare (control) and T₀ plants of OsTDL1A-RNAi line #4363.

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