The Rice Wall-Associated Receptor-Like Kinase Gene OsDEES1 Plays a Role in Female Gametophyte Development

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The wall-associated kinase (WAK) gene family is a unique subfamily of receptor-like kinases (RLKs) in plants. WAK-RLKs play roles in cell expansion, pathogen resistance, and metal tolerance in Arabidopsis (Arabidopsis thaliana). Rice (Oryza sativa) has far more WAK-RLK genes than Arabidopsis, but the functions of rice WAK-RLKs are poorly understood. In this study, we found that one rice WAK-RLK gene, DEFCET IN EARLY EMBRYO SAC1 (OsDEES1), is involved in the regulation of early embryo sac development. OsDEES1 silencing by RNA interference caused a high rate of female sterility. Crossing experiments showed that female reproductive organs lacking OsDEES1 carried a functional defect. A detailed investigation of the ovaries from OsDEES1 RNA interference plants indicated that the knockdown of OsDEES1 expression did not affect megasporogenesis but that it disturbed female gametophyte formation, resulting in a degenerated embryo sac and defective seed formation. OsDEES1 exhibited a tissue-specific expression pattern in flowers and seedlings. In the ovary, OsDEES1 was expressed in the megagametophyte region and surrounding nucellus cells in the ovule near the micropylar region. OsDEES1 was found to be a membrane-localized protein with a unique sequence compared with other WAK-RLKs. These data indicate that OsDEES1 plays a role in rice sexual reproduction by regulating female gametophyte development. This study offers new insight into the functions of the WAK-RLK family.

Receptor-like kinases (RLKs) comprise a plant superfamily with a greater number of genes than in similar protein superfamilies in animals. The dramatic expansion of this family in plants is postulated to be crucial for plant-specific adaptations (Lehti-Shiu et al., 2009). A typical RLK contains a signal sequence, a transmembrane region, and a C-terminal domain with eukaryotic protein kinase signatures (Shiu et al., 2004). The cell wall-associated kinase (WAK) family is a unique subfamily of plant RLKs in which the extracellular region physically binds the cell wall (He et al., 1996; Verica et al., 2003). Typical WAK-RLKs are transmembrane proteins with a cytoplasmic Ser/Thr kinase domain and an extracellular region with several vertebrate epidermal growth factor (EGF)-like domains (Verica and He, 2002). The EGF repeat is the only motif found to date in both plant RLKs and animal receptor Tyr kinases (Shiu and Bleecker, 2001). This motif is thought to participate directly in protein-protein interactions in animals, but its function in plants is undetermined. Because of their unique structural characteristics, WAK-RLKs are thought to be good candidates for transferring signals from the cell wall to the cytoplasm; the extracellular domain can sense stimuli at the cell wall and transmit signals inside the cell via the cytoplasmic kinase domain (Anderson et al., 2001; Shiu and Bleecker, 2001; Verica et al., 2003). The functions of WAK-RLKs in plants have been extensively studied in Arabidopsis (Arabidopsis thaliana). Arabidopsis WAK-RLKs have been shown to play roles in the regulation of cell expansion (Lally et al., 2001), resistance to pathogenic bacteria (He et al., 1998), and tolerance to heavy metals (Hou et al., 2005). Although functional analyses of WAK-RLKs are incomplete, a few reports suggest that they act as signal messengers in plant cells. It was reported that AtWAK1 interacts with cell wall-localized molecules, including a Gly-rich secreted protein (AtGRP3), pectin, polygalacturonic acid, and oligogalacturonides, via its extracellular domain (Park et al., 2003; Decreux and Messiah, 2005). Furthermore, AtWAK1 has been reported to act as an oligogalacturonide receptor, with binding occurring through its extracellular domain (Brutus et al., 2010). Recently, it was reported that the intracellular domain of AtWAK1L10 has both guanylyl

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1 This work was supported by the National Key Program for the Development of Basic Research in China (grant no. 2013CB126903), the National Science Foundation of China (grant nos. 30770216 and 31170292), the Natural Science Foundation of Hebei Province in China (grant no. C2010000390), and the National Program of High Technology Development of China (grant nos. 2008ZX08009–003).

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[1] Some figures in this article are displayed in color online but in black and white in the print edition.

[2] The online version of this article contains Web-only data. www.plantphysiol.org/cgi/doi/10.1104/pp.112.203943
cyclase and kinase activity, indicating that AtWAK1 is a twin-domain catalytic protein (Meier et al., 2010).

The number of WAK-RLK genes in rice (*Oryza sativa*) is far greater than that in Arabidopsis. A total of five WAK genes and 21 WAK-like genes have been identified in the Arabidopsis genome (Verica et al., 2003). In rice, through a reiterative database search and manual reannotation, 125 WAK-like genes were identified; of these, 67 were rice WAK-RLKs (OsWAKRLKs; Zhang et al., 2005). This expansion of OsWAK-RLKs may be due to lineage-specific expansion of the family in monocots (Zhang et al., 2005). However, the functions of OsWAK-RLKs are largely unknown, except for OsWAK1, a novel WAK-RLK gene in rice with reported roles in plant disease resistance (Li et al., 2009). To date, there have been no reports on the function of OsWAK-RLKs in rice development or growth.

Based on expression data from a microarray study of floral development in rice, we selected a number of candidate OsWAK-RLK genes whose expression levels were high or significantly changed in flowers at the heading stage to investigate their functions in rice sexual reproduction. The expression products of these genes were predicted to be localized to the plasma membrane and to have the typical structure of WAK-RLKs. In this report, we describe one of the selected WAK-RLK genes, DEFECT IN EARLY EMBRYO SAC1 (*OsDEES1*), which is involved in the regulation of rice reproduction. The knockdown of *OsDEES1* expression by RNA interference (RNAi) caused sterility in transgenic rice plants. Further study showed that the female reproductive unit (embryo sac) was seriously malformed and sterile, resulting in a lack of seed setting.

**RESULTS**

**OsDEES1** Interference Results in High Rates of Sterility

To investigate *OsDEES1* function, we knocked down *OsDEES1* expression by RNAi. A specific 426-bp fragment (243–668) from the N terminus of *OsDEES1* complementary DNA (cDNA) was inserted into the RNAi vector pTCK303 (Wang et al., 2004) and then introduced into rice by *Agrobacterium tumefaciens* EHA105-mediated transformation. The RNAi transgenic rice exhibited a near-normal morphology in terms of growth but a high rate of sterility (Fig. 1, A and B). Of 44 RNAi transgenic plants obtained over three years (2005, 2006, and 2007), 33 had high rates of sterility (Fig. 1C). For example, in 2005, the sterility of six RNAi plants ranged from 73% to 100%, compared with 27% and 9% in the empty vector–only transgenic control and wild-type plants, respectively. In 2006 and 2007, a similar phenotype was observed; the seed yield of most T0 RNAi plants was very low compared with that of wild-type and vector control plants grown under the same conditions (Fig. 1C). The height (Supplemental Fig. S1A), tiller number (Supplemental Fig. S1B), and culm compact (Supplemental Fig. S1C) in the RNAi plants were not obviously affected, except that the elongated uppermost internode in the *OsDEES1* RNAi plants was shorter than that in the control plants (Supplemental Fig. S1D). Southern-blot analysis showed that most of the T0 RNAi plants were unique transgenic lines (Supplemental Fig. S2). Among these plants, lines 3, 5, 73, and 74 were selected as representative *OsDEES1* RNAi plants for detailed analysis. The four representative *OsDEES1* RNAi transgenic lines were propagated by resistance selection, and the offspring exhibited a similar sterile phenotype from the T1 (Supplemental Fig. S3A) to the T4 generation (data not shown). A semiquantitative reverse transcription (RT)-PCR assay revealed that the mRNA expression of endogenous *OsDEES1* was significantly down-regulated in our RNAi plants (Fig. 1D; Supplemental Fig. S3B) and that the expression of the target gene was consistent with the level of sterility in the T0 RNAi plants. Strong interference lines such as 2-6 and 67-80 exhibited a high sterility rate, whereas weak interference lines such as 82 and 85 were associated with a low sterility rate (Fig. 1C). To determine whether the interference of *OsDEES1* also affected its rice homologs, the expression of *OsDEES1-L4* (Os09g0562600), which possessed a homologous sequence with the *OsDEES1* RNAi fragment and which exhibited a tissue expression pattern similar to that for *OsDEES1* (Supplemental Fig. S4), was tested in the *OsDEES1* RNAi plants. RT-PCR analysis showed that the expression of *OsDEES1-L4* was not affected in the RNAi plants, indicating that the RNAi was specific against *OsDEES1* in our transgenic plants (Fig. 1E). These data indicate that the knockdown of *OsDEES1* expression caused sterility in the RNAi transgenic plants.

Seed Development Was Arrested at the Fertilization Stage in the RNAi Plants

To prove that the sterility of our RNAi transgenic plants resulted from reproductive organ abnormalities, floral and caryopsis phenotypes were carefully examined throughout the reproductive period. Up to the fertilization stage, the *OsDEES1* RNAi flowers showed no morphological differences from the controls: the glumes, anther, stigma, and pistil looked normal compared with wild-type flowers (Fig. 2A, left). However, after fertilization, seed expansion and elongation, which were observed in wild-type plants, did not occur in the *OsDEES1* RNAi plants. At the early filling stage, no seed development could be observed in the RNAi plants because the ovules did not enlarge after pollination (Fig. 2A, middle). At the late filling stage, the seeds in the wild-type plants developed to the maximum size, whereas the *OsDEES1* RNAi ovules remained at the size seen at the fertilization stage (Fig. 2A, right). Statistical analysis showed that at the end of the filling stage, the rate of abortion in the RNAi
caryopses ranged from 67% to 90%, which is significantly higher than the 6% abortion rate detected in the wild type (Fig. 2B). These data are in accordance with the rate of sterility (58%–86%) in the T1 seeds (Supplemental Fig. S3). This result indicates that seed development in the OsDEES1 RNAi plants was arrested at the fertilization stage.

OsDEES1 RNAi Plant Sterility Is the Result of Female Reproductive Organ Defects

Fertilization requires functional male and female gametophytes, which are produced by the stamen and pistil, respectively. To determine whether the male or female reproductive organs in our RNAi plants had functional defects, we performed artificial pollination experiments between wild-type and OsDEES1 RNAi plants. Crossing experiments were performed at least two times in 2008 and 2009. Two or three representative crosses are shown in Table I. Our results show that offspring from the control cross (WT♀ × WT♂) exhibited 73% to 89% fertility. The pollination of wild-type pistils with pollen from RNAi plants produced similar fertility rates, ranging from 50% to 85% fertility in line 3, 75% to 78% fertility in line 73, and 39% to 82% fertility in line 74 (Table I). However, when wild-type pollen was used to pollinate RNAi pistils, the offspring (WT♀ × RNAi♂) produced very few grains: three plants had 0% fertility, 73 plants had 0% to 11% fertility, and 74 plants had 0% to 10% fertility (Table I). Taken together, these results suggest that the pollen from the OsDEES1 RNAi plants was functional and that the sterility of the OsDEES1 RNAi plants might be mainly due to defects in the female reproductive organs.

Male Gametophyte Development Is Moderately Affected in OsDEES1 RNAi Plants

Although the above results suggested that defects in the female gametophyte led to abortion of the rice, whether the male gametophyte was affected in our OsDEES1 RNAi plants was unclear. Thus, we analyzed the pollen viability and germination capability in
our transgenic lines. Pollen viability was examined by iodine-potassium iodide (I2-KI) and triphenyl tetrazolium chloride (TTC) staining. As shown in Figure 3, the number of well-stained pollen grains in the OsDEES1 RNAi plants was less than that in wild-type and vector control transgenic plants (Fig. 3A). Statistical analysis showed that the inviable rate for the RNAi pollen was 8% to 23%, which is slightly higher than that in the wild-type (2%) and control transgenic (4%) plants (Fig. 3B). To determine whether the decrease in pollen viability in the RNAi plants would affect pollen germination and elongation, we analyzed pollen germination on the stigma and pollen tube growth in the ovary in vivo. In wild-type plants, 86% of the pollen could germinate on the stigma and the pollen tube could grow to the ovule micropyle, whereas this figure decreased slightly to 80% to 84% in the OsDEES1 RNAi plants (Fig. 3, C and D). This result indicates that the knockdown of OsDEES1 expression moderately affected pollen viability and pollen tube growth without affecting overall plant fertility.

**OsDEES1 Interference Disturbs Female Gametophyte Development**

The results of our crossing experiments suggested that defective female organ development in the OsDEES1 RNAi plants contributed to the observed sterility. To test this hypothesis, we examined female gametophyte formation and development by the microscopic observation of semithin sections and confocal microscopy. Embryo sac development in our RNAi plants was completely normal at the megasporogenesis stage but was seriously affected at the megagametogenesis stage. The megaspore mother cell developed through the dyad and tetrad stages and formed a functional megaspore in both wild-type (Fig. 4A, a–d; Supplemental Fig. S5, A–C) and OsDEES1 RNAi (Fig. 4A, e–h; Supplemental Fig. S5, F–H) plants. However, during megagametogenesis, formation and development of the female gametophyte seemed to be disturbed in the OsDEES1 RNAi plants. The RNAi functional megaspore was normal, but the newly formed embryo sac showed severe defects. At the middle stage of female gametophyte development, when the embryo sac should develop into a coenocyte with four nuclei in the wild type, RNAi gametophyte development was found to be arrested at the functional megaspore stage (Fig. 4Am). At the late stage of

![Figure 2. Floral morphology and caryopsis development in the OsDEES1 RNAi and wild-type plants (WT). A, Flowers and caryopses from wild-type and RNAi plants at different stages of reproduction. B, Statistical analysis of the caryopsis abortive rate at the end of the filling stage. [See online article for color version of this figure.]](image)

**Table 1. Seed setting and fertility in the offspring of the crosses between OsDEES1 RNAi and wild-type plants**

<table>
<thead>
<tr>
<th>Cross (♀×♂)</th>
<th>Fertile Seeds</th>
<th>Sterile Seeds</th>
<th>Total Seeds</th>
<th>Fertility</th>
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<tr>
<td>WT×WT</td>
<td>1 25</td>
<td>3 28</td>
<td>89</td>
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<tr>
<td>WT×3</td>
<td>2 36</td>
<td>13 49</td>
<td>73</td>
<td></td>
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<tr>
<td>3×WT</td>
<td>1 23</td>
<td>4 27</td>
<td>85</td>
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<tr>
<td>2 34</td>
<td>16 50</td>
<td>68</td>
<td></td>
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<tr>
<td>3 18</td>
<td>18 36</td>
<td>50</td>
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<tr>
<td>3×WT</td>
<td>1 0</td>
<td>44 44</td>
<td>0</td>
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<td>2 0</td>
<td>26 26</td>
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<td>3 0</td>
<td>21 21</td>
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<tr>
<td>WT×73</td>
<td>1 45</td>
<td>13 57</td>
<td>78</td>
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<td>2 42</td>
<td>13 55</td>
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<td>3 36</td>
<td>12 48</td>
<td>75</td>
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<tr>
<td>73×WT</td>
<td>1 6</td>
<td>46 52</td>
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<td>2 0</td>
<td>45 45</td>
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<td>3 0</td>
<td>46 46</td>
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<tr>
<td>WT×74</td>
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<td>7 40</td>
<td>82</td>
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female gametophyte development, when an embryo sac with eight nuclei should be produced, the microscopic observation of semithin sections revealed that the germ cells in the RNAi plants appeared to have degenerated, resulting in the formation of embryo sacs with various abnormalities, including completely degenerated embryo sacs, small and deformed embryo sacs, and empty embryo sacs without germ cells (Fig. 4A, n–p). During the final stage of embryo sac development, two types of abnormalities in the embryo sacs were observed: either the degenerated embryo sac disappeared completely and was filled with ovule nucellus cells (Supplemental Fig. S5J1) or the defective embryo sac had an abnormal or degenerated female germ unit (Supplemental Fig. S5J2). In contrast, wild-type embryo sac development proceeded normally, forming a mature embryo sac with antipodal, central cells, and an egg apparatus (Fig. 4A, i–l; Supplemental Fig. S5, A–E).

We also examined the development of fertilized ovaries in our RNAi plants by confocal microscopy. At 24 to 48 h post fertilization, we observed a multicellular globular embryo at the micropylar end and free endosperm nuclei suspended in the peripheral cytoplasm of the embryo sacs in the wild type (Fig. 4B). In comparison, in the OsDEES1 RNAi plants, fertilization appeared not to have occurred; most embryo sacs were still degenerated or empty and no globular embryo or endosperm nuclei could be found (Fig. 4B). This indicates that the abnormal embryo sac might have lost its capacity for fertilization, resulting in a lack of seed setting in the OsDEES1 RNAi plants. These data show a significant increase in the frequency of abnormal (empty and degenerated) embryo sacs in the OsDEES1 RNAi plants (60.8%–82.5% compared with 14.7% in the wild type; Fig. 4C). These data are consistent with the rates of sterility in our OsDEES1 RNAi and wild-type plants (Fig. 1C; Supplemental Fig. S3). Therefore, our data demonstrate that OsDEES1 RNAi caused defects in embryo sac formation, resulting in the production of degenerated or empty embryo sacs and, ultimately, sterility.

The OsDEES1 Expression Pattern during Plant Growth and Reproduction

To investigate the tissue-specific expression pattern of OsDEES1, a 1.8-kb promoter sequence was constructed using a modified pCAMBIA1300 vector harboring the gene encoding GUS. This OsDEES1pro::GUS construct was induced into rice plants by A. tumefaciens-mediated callus transformation. Histochemical staining revealed OsDEES1 expression in floral tissues (anthers, pistils, and lodicules) as well as in the coleoptile, node, and leaf tongue of seedlings, but not in the leaf blade or root (Fig. 5A).

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plants were generated for immunohistochemical analysis using anti-GUS antibodies. As shown in Figure 5B, a strong GUS immunolocalization signal (brown) was detected in the ovule and lodicules (Fig. 5Bb). In the enlarged image of the ovule, OsDEES1 expression can be seen in the megagametophyte region and surrounding nucellus cells near the micropyle (i.e. the area where the embryo sac formed and developed; Fig. 5Bc). In comparison, in the negative control, only the background signal (blue) was detected (Fig. 5Ba). Therefore, the expression pattern of OsDEES1 is consistent with our genetic data, which strongly suggest that OsDEES1 plays a critical role in embryo sac development.
OsDEES1 Is a Unique Member of the WAK-RLK Gene Family

Online sequence analyses have shown that OsDEES1 possesses a typical receptor kinase structure, including an extracellular domain, a transmembrane domain, and an intracellular kinase domain (http://plantsp.genomics.purdue.edu/feature_scan.html). We made a detailed analysis of the functional domains in OsDEES1 and found that the extracellular region has a signal peptide composed of 19 amino acids at the N terminus and two EGF repeat-like domains (EGF2 and EGF-Ca) near the transmembrane region. The typical ATP-binding motif and kinase active site can also be found in the intracellular domain (Fig. 6A). Phylogenetic tree analysis showed that OsDEES1 shares 41% identity with three Arabidopsis WAKs (AtWAK1, -2, and -4), whereas the three AtWAKs exhibited approximately 73% identity among themselves (Fig. 6B). Notably, compared with AtWAKs, OsDEES1 possesses an additional 42 amino acids in its extracellular domain but lacks 63 amino acids at its C terminus (Fig. 6A). A reiterative database search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and manual reannotation revealed that the rice genome possesses four OsDEES1 homologs (OsDEES1-L1 to -4). Interestingly, it also showed a deletion of more than 60 amino acids in the C-terminal region of OsDEES1 compared with its homologs (Supplemental Fig. S4A). A protein sequence alignment indicated that OsDEES1 and its homologs exhibited about 66% to 93% identity (Supplemental Fig. S4B). However, OsDEES1 and its homologs had different expression patterns. RT-PCR analysis showed that OsDEES1 and OsDEES1-L4 were predominantly expressed in flowers; in comparison, OsOsDEES1-L1 was expressed in roots, leaves, and flowers, while OsDEES1-S2 and OsDEES1-L3 were mostly expressed in roots (Supplemental Fig. S4C). These data suggest that OsDEES1 is distinct from other members of the WAK-RLK gene family.

The OsDEES1 gene product has a membrane-localizing signal peptide and is thus speculated to be a plasma membrane protein (http://cdna01.dna.affrc.go.jp/cDNA/). To verify this, a fusion protein, OsDEES1ΔK-YFP (OsDEES1ΔK refers to OsDEES1 with a kinase domain deletion, and YFP is yellow fluorescent protein), was generated and expressed.
transiently under the control of the 35S promoter in tobacco (Nicotiana tabacum) and onion (Allium cepa) epidermal cells. As shown in Figure 6C, YFP alone was expressed ubiquitously in the intracellular region of the tobacco epidermal cells, whereas OsDEES1ΔK-YFP was detected only in the region of the plasma membrane. In onion epidermal cells, which were treated with 0.9 M mannitol to induce plasmolysis, OsDEES1ΔK-YFP was detected in the plasma membrane region and in the cell wall region as well as in some thread-like structures between the cell wall and plasma membrane. In contrast, the control protein (YFP) was detected only in the intracellular region (Fig. 6C). These data indicate that OsDEES1 is plasma membrane localized and that it may connect tightly with the cell wall, resulting in its extracellular localization after plasmolysis. Together, these data suggest that OsDEES1 is a wall-associated RLK.

DISCUSSION

OsDEES1 Plays a Role in Rice Fertility as a Unique WAK-RLK Gene

The WAK-RLK subfamily is a plant-specific RLK superfamily in which the extracellular domain is covalently bonded to the cell wall (He et al., 1996; Verica et al., 2003). Typical WAK-RLKs possess an extracellular domain containing EGF repeat domains, a transmembrane region, and an intracellular domain carrying a typical eukaryotic Ser/Thr kinase signature (Shiu et al., 2004). There are 26 WAK-RLK genes in...
Arabidopsis (He et al., 1999; Verica et al., 2003) and 125 in rice (Zhang et al., 2005). It was previously shown that the OsWAK-RLK subfamily is greatly expanded in rice, but the functions and protein structural characteristics of these putative genes are unknown (Zhang et al., 2005). The biological roles of WAK-RLKs have largely been elucidated from studies in Arabidopsis. AtWAK-RLK family members function in cell expansion, resistance to pathogenic bacteria, and tolerance to heavy metals (He et al., 1998; Lally et al., 2001; Hou et al., 2005). In Arabidopsis, a reduction in the expression of all five AtWAK genes (AtWAK1 to -5) is lethal, implying that the WAK-RLK family plays fundamental roles in plant development (Lally et al., 2001).

Here, we provide evidence that a unique OsWAK-RLK gene, OsDEES1, is involved in rice fertility. The knockdown of OsDEES1 expression by RNAi resulted in high rates of sterility (Fig. 1). This phenotype was observed in 33 individual OsDEES1 RNAi transgenic lines, which were generated separately over three years (2005, 2006, and 2007), and it was passed on to offspring plants carrying the RNAi construct (Supplemental Fig. S3). In these transgenic plants, OsDEES1 RNAi was very effective and specific, and the decrease in OsDEES1 expression was consistent with the degree of sterility (Fig. 1; Supplemental Fig. S3). We also generated OsDEES1-overexpressing plants, but they showed no differences in fertility or morphology compared with the wild type (data not shown). Southern analysis indicated that most of our RNAi transgenic plants were independent T-DNA insertion lines (Supplemental Fig. S2). That the same sterility phenotype was observed in our multiple RNAi transgenic lines indicates that OsDEES1 plays an important role in rice fertility.

In this study, OsDEES1 was shown to possess similar structural features with the WAK-RLK family, including an extracellular region with two EGF repeats, a transmembrane domain, an intracellular region with a eukaryotic protein kinase signature, and an extracellular domain that is tightly connected with the cell wall (Fig. 6). Interestingly, there were also some distinct features in OsDEES1. For example, OsDEES1 does not have a conserved C-terminal sequence compared with Arabidopsis WAK-RLKs (Fig. 6) and its rice homologs (Supplemental Fig. S3). Moreover, the tissue-specific expression pattern of OsDEES1 in rice contrasts sharply with the nearly ubiquitous expression of AtWAKs in normal developing tissues in Arabidopsis (Lally et al., 2001; Wagner and Kohorn, 2001). In rice, OsDEES1 also has a distinctive expression pattern compared with its homologs (Supplemental Fig. S4C). Thus, OsDEES1 is a unique WAK gene. The function of OsDEES1 in rice reproduction adds a new role to the growing list of functions of the WAK-RLK subfamily in plant growth and development.

OsDEES1 Is Involved in the Regulation of Embryo Sac Development

Sexual reproduction in rice consists of floral organ formation and maturation, fertilization, and caryopsis development. Observation of the entire reproductive period showed that seed development was arrested at the fertilization stage in OsDEE1 RNAi plants (Fig. 2). Crossing experiments showed a functional defect in the female reproductive organs of the RNAi plants (Table I). By the microscopic observation of semithin sections and confocal microscopic studies of whole-mount cleared ovaries, we found that the sterility caused by the knockdown of OsDEES1 expression was due to defects in female gametophyte formation and development. OsDEES1 RNAi disturbed embryo sac formation after the functional megaspore stage and resulted in abnormal embryo sac development (Fig. 4, A and B), which ultimately caused plant sterility. The expression of OsDEES1 in the ovule was consistent with its function (Fig. 5B). Therefore, OsDEEES1 is involved in the regulation of female gametophyte development in rice.

The female gametophyte is critical for plant reproduction. Female gametophyte development begins early in ovule development, with the formation of a diploid megaspore mother cell that undergoes meiosis. One resulting haploid megaspore then develops into the female gametophyte (Drews and Koltunow, 2011). The female gametophyte is a multicellular haploid structure that produces the embryo and endosperm after fertilization; thus, it has become an attractive model system for investigating the molecular mechanisms of nucleus migration, cell specification, cell-to-cell communication, and many other processes (Ling et al., 2012). Development of the haploid female gametophyte must be coordinated with that of the surrounding sporophytic integuments (Yang and Sundaresan, 2000; Acosta-Garcia and Vielle-Calzada, 2004). Cell-cell interactions or signaling transductions between the developing female gametophyte and neighboring nucellus and the resulting signaling cascades are important for the establishment of polarity and cell fate determination in female gametophyte development (Bajon et al., 1999; Yadegari and Drews, 2004).

So far, only a few genes have been reported to be involved in the regulation of signal transduction during female gametophyte development. For example, the asymmetric distribution of the hormone auxin has been reported to control the polarity of female gametophytes by regulating cell fate specification and cellularization in Arabidopsis (Pagnussat et al., 2009). Arabinogalactan protein18, which is abundant in the cell wall, is essential for the initiation of female gametogenesis in Arabidopsis (Acosta-Garcia and Vielle-Calzada, 2004). It is known that the hydrophilic molecular regulon outside the cell transfers the signal across the plasma membrane through a membrane-localized receptor. RLKs are thought to be good
candidates for transferring the signal across the plasma membrane (Morris and Walker, 2003). In Arabidopsis, a leucine-rich repeat (LRR) RLK gene, EMS1/EXS, regulates male germ line cell number and tapetal identity (Canales et al., 2002; Zhao et al., 2002). In rice, *MULTIPLE SPOROCYTES1* (MSPI), an LRR receptor kinase and EMSI/EXS homolog, regulates the number of cells entering into sporogenesis both in male and female organs (Nonomura et al., 2003). A small protein, OsTDL1A, which is very similar to TPD1, binds the LRR domain of MSPI and plays roles in controlling the number of sporocytes (Zha et al., 2008). Here, we demonstrated that a wall-associated RLK gene, OsDEES1, is critical for female gametophyte formation. LRR-RLKs and WAK-RLKs differ mainly in their functional domains (LRR versus EGF-like) located in the extracellular region. LRR and EGF-like domains are thought to participate in protein-protein interactions. In addition, WAK-RLKs can physically bind to the cell wall (He et al., 1996). MSPI functions in male and female sporogenesis in cooperation with its ligand, OsTDL1A, whereas OsDEES1 plays a role mainly in female gametogenesis. The dynamic phase transition from sporophyte to gametophyte requires communication between germ cells and the nucellus cells of the ovule (Yadegari and Drews, 2004). During female gametophyte development, embryo sac development requires highly synchronized morphogenesis of the maternal sporophyte surrounding the gametophyte (Bencivenga et al., 2011). Interestingly, OsDEES1 was expressed in the gametophyte region and surrounding ovule nucellus cells (Fig. 5B), so we may presume that OsDEES1, as a unique wall-associated RLK, participates in the complex signaling cascades of female gametophyte development by mediating signals between the gametophyte and surrounding cells. Given the flower-specific expression and membrane localization of OsDEES1, we propose a working model in which OsDEES1 senses the signal from the cell wall or other extracellular molecule, which is critical for normal female gametophyte development, and transduces it into the cell to coordinate embryo sac development. However, further study is necessary to understand the detailed mechanisms underlying the regulatory effects of OsDEES1 on embryo sac development.

MATERIALS AND METHODS

**Plant Materials and Growth Conditions**

All wild-type rice (*Oryza sativa* 'Nipponbare') and transgenic plants were grown in a greenhouse at 24°C to 30°C (16 h of light/8 h of darkness) for 1 month and then transferred to a field and planted under natural conditions until harvest. Self-pollinated seeds from independent transgenic lines were selected on Murashige and Skoog medium containing 4 mg L\(^{-1}\) 2,4-dichlorophenoxyacetic acid for 4 weeks in the dark at 28°C to induce embryogenic calli for *A. tumefaciens*-mediated transformation. The positively transformed calli were selected using hygromycin B (50 mg L\(^{-1}\)) and differentiated on differentiation medium (0.5 mg L\(^{-1}\) naphthalacetic acid and 3 mg L\(^{-1}\) 6-benzyladenine) to generate T0 seedlings.

**Phenotype Characterization**

Photographs of spikelets and whole plants were taken from transgenic rice grown in the field. The statistical analysis of sterility was done as follows. Five panicles were randomly selected from each transgenic plant, and the number of caryopses was determined. The sterility rate was then calculated as unfertilized caryopses (shrunken grains)/all caryopses (shrunken grains plus full grains) × 100%. The abortion rate during caryopsis development was analyzed as follows. Three spikelets were randomly cut from RNAi transgenic rice at the filling stage, their glumes were removed, and the numbers of normal caryopses (expanded seeds) and abortive caryopses were used to calculate the abortion rate.

**Southern Blotting**

Southern blotting was used to identify T-DNA insertions in the genome of the transgenic rice plants (Sambrook et al., 1989). Genomic DNA extracted from 40-d-old seedlings was digested with *HindIII* (Takara), separated by 0.8% agarose gel electrophoresis, transferred to a Nylon+ membrane (Gelman), and then hybridized with a \([^{32}P]\)DNA probe. In this experiment, a hygromycin fragment labeled with \([^{32}P]\)CTP was used as the hybridization probe; pTCK303 was used as a positive control plastid; and wild-type rice 'Nipponbare' plants were used as negative controls (Sambrook et al., 1989). Images were produced using Typhoon 9200.

**RNA Level Analysis**

Semi-quantitative RT-PCR was performed to analyze the interference effect of OsDEES1. Total RNA was isolated from the panicles. One microgram of total RNA was reverse transcribed using AMV Reverse Transcriptase XL (Takara) with oligo(dT)\(_{12-18}\) primer. OsDEES1-specific primers (forward, 5′-GCTCTCGAG-CAGCGCAACC-3′; reverse, 5′-CAGCGCCAAACC-3′; reverse, 5′-CACTGCCAATCCTCAAAATC-3′) were used to amplify a fragment (800 bp) of cDNA with an intron inside. An OsDEES1-4′-specific fragment was amplified by RT-PCR using the forward primer 5′-GACCGCAAAACAGCAAG-3′ and reverse primer 5′-GATGACTCTCGG-CGAAGA-3′. OsActin1 mRNA was used as a standard control, and a 600-bp cDNA fragment was amplified using OsActin-specific primers (forward, 5′-CCCTCTCAGCCCTCTGCAGG-3′; reverse, 5′-GACGAAACCGAGAGC-CGCC-3′). RT-PCR was performed in an Eppendorf thermocycler with the following cycling profile: 94°C for 5 min; followed by 26 to 33 cycles of 94°C for 30 s, 60°C for 40 s, and 72°C for 1 min; with a final extension at 72°C for 10 min.

**Embryo Sac Analysis**

For semithin sectioning and microscopy, florets were collected at different stages of female gametophyte development according to the length of the flower. The ovaries were treated for 30 min in 0.05% acetic acid, washed three times in sterile water, fixed in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4), postfixed in 1% osmium tetroxide (pH 7.4) for 2 h, dehydrated in a graded ethanol series, and polymerized at 55°C for 48 h. Semithin sections (5 μm) were cut with a rotary microtome and stained with lead citrate and uranyl blue O.

Confocal microscopy was carried out according to the whole-mount eosin B-staining confocal laser scanning microscopy method (Zeng et al., 2007). The fixed materials were hydrated using gradient ethanol solutions, pretreated with 2% aluminum potassium sulfate for 20 min, and then stained with 10 mg L\(^{-1}\) eosin B (C\(_{16}\)H\(_{22}\)N\(_{2}\)O\(_{4}\)Br\(_{2}\)Na\(_{2}\)) for 10 to 12 h at room temperature. The
samples were then posttreated in 2% aluminum potassium sulfate for 20 min and dehydrated using a series of ethanol solutions (30%, 50%, 70%, 90%, and 100% v/v). The cleared ovaries were scanned with a laser scanning confocal microscope (Zeiss LSM510). The excitation wavelength was 543 nm; emitted light was detected at 550 to 630 nm.

**Pollen Viability and Germination Assays**

We used Lk-KI staining to evaluate pollen viability. Anthers were placed in Lk-KI staining buffer containing 1% (w/v) Lk in 3% (w/v) KI and 1% (w/v) TTC buffer with vibration for 20 to 30 min at room temperature to stain the released pollen. Pollen grains that were round in shape and stained black by Lk-KI were judged as viable or living, while those that stained yellow or light red were judged as sterile or dead. Pollen grains stained red or pink by TTC were judged as viable, while those that appeared gray or colorless were deemed sterile.

Pollen germination and pollen tube growth were examined by aniline blue staining as described (Chhun et al., 2007) with modifications. Rice flowers were emasculated and artificially pollinated by hand. After 30 min (germination assay) or 4 h (pollen tube growth assay), the pistils were excised, fixed in 3:1 ethanol:acetic acid for 30 min, softened in 1N KOH for 20 h, and then sections were then covered with rabbit anti-mouse IgG coupled to horseradish peroxidase as described (Jun et al., 2004) or transformed into onion (Allium cepa) epidermal cells by particle bombardment using the Bio-Rad PDS-1000/He system according to the manufacturer’s protocol. Localization of the protein was examined after 40 h at room temperature. The pistils were then rinsed briefly in distilled water and mounted in 50% glycerol. The samples were visualized by UV microscopy.

**OsDEES1 Expression and Histological Assays**

Two fragments containing 1.8 kb of the upstream region of OsDEES1 were amplified by PCR using the forward primer 5'-ACATGCAGCTGGACACCT-CAAGGTGTATCCCAGT-3’ and reverse primer 5'-GGATCCCTGCTGGAACATAGT-3’. The amplified fragment was inserted into modified pCAMBIA1300 to drive GUS expression using the HindIII and Xhol restriction sites. The resulting construct, OsDEES1pro::GUS, was transformed into wild-type rice. GUS staining was performed as follows. Samples from different tissues of OsDEES1pro::GUS transgenic lines were vacuum infiltrated with 50 mM Na3PO4, pH 7.0, 10 mg mL-1 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid, and 0.02% Triton X-100 and then incubated at 37°C for 12 h. After staining, the organs were destained in 70% ethanol several times until the chlorophyll was removed.

Immunolocalization of OsDEES1 was performed as described by McGraw et al. (2007) with a modification. Ovarian samples from OsDEES1pro::GUS transgenic plants were fixed in formaldehyde:acetic acid:50% ethanol (5:6:89, v/v) and mounted in parafill blocks. The slides were then incubated for 2 h at room temperature in the presence of polyclonal anti-GUS antibodies. The tissues were then covered with rabbit anti-mouse IgG coupled to horseradish peroxidase (EnVision), and the immune complex was revealed using 3,3'-diaminobenzidine (brown). The tissues were stained with hematoxylin (blue), and then the slides were observed and photographed by light microscopy.

**Subcellular Localization of OsDEES1**

To investigate the subcellular localization of OsDEES1, we constructed the vector CaMV35S::WRKYK-YFP. A PCR fragment of OsDEES1 cDNA lacking the kinase region was amplified using specific primers (forward, 5’-GCTCTAGAAACCCGCGTATGCCTGAGT-3’; reverse, 5’-CGGATCCGGCCCTTCACATCTGCAAGAGG-3’) containing Xhol and BamHI sites, respectively. The products were cloned into modified pCAMBIA1300 to create CaMV35S::OsDEES1pro::GUS. CaMV35S::YFP was used as a negative control vector. Both vectors were transferred to A. tumefaciens strain GV3101 and then separately and transiently transformed into tobacco (Nicotiana tabacum) leaves (Jun et al., 2004) or transformed into onion (Allium cepa) epidermal cells by particle bombardment using the Bio-Rad PDS-1000/He system according to the manufacturer’s protocol. Localization of the protein was examined after 40 to 48 h using a Zeiss LSM 510 confocal microscopy system with a 514-nm laser for excitation and 530 to 540 nm for YFP emission.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers OsDEES1 (Os09g0561600), OsDEES1-L4 (Os09g0562600), and OsActin1 (Os03g0718100).

**Supplemental Data**

The following materials are available in the online version of this article.

**Supplemental Figure S1.** Statistical analysis of the height, tiller number, culm compact, and uppermost internode length in the OsDEES1 RNAi transgenic rice plants.

**Supplemental Figure S2.** Southern-blot assay for the T-DNA insertions in the T0 OsDEES1 RNAi transgenic plants by autoradiography.

**Supplemental Figure S3.** Sterility rate and OsDEES1 expression level in the T1 RNAi transgenic plants.

**Supplemental Figure S4.** Analysis of the OsDEES1 homologs in rice.

**Supplemental Figure S5.** Observation of the female gametophyte in OsDEES1 RNAi plants by confocal microscopy.

**Supplemental Figure S6.** Analysis of OsDEES1 expression during reproduction.

**ACKNOWLEDGMENTS**

We thank Dr. Wen-Qiang Tang at the Institute of Molecular Cell Biology of Hebei Normal University for insightful suggestions and comments on the manuscript and Dr. Kang Chong at the Institute of Botany of the Chinese Academy of Sciences for providing the pTC3K03 vector. Received July 19, 2012; accepted August 7, 2012; published August 10, 2012.

**LITERATURE CITED**


OsDEES1 Plays a Role in Female Gametophyte Development


