Processes Underlying a Reproductive Barrier in \textit{indica-japonica} Rice Hybrids Revealed by Transcriptome Analysis

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In rice (\textit{Oryza sativa}), hybrids between \textit{indica} and \textit{japonica} subspecies are usually highly sterile, which provides a model system for studying postzygotic reproductive isolation. A killer-protector system, \textit{S}5, composed of three adjacent genes (\textit{ORF3}, \textit{ORF4}, and \textit{ORF5}), regulates female gamete fertility of \textit{indica-japonica} hybrids. To characterize the processes underlying this system, we performed transcriptomic analyses of pistils from rice variety Balilla (BL), Balilla with transformed \textit{ORF5} (BL5+ producing sterile female gametes, and Balilla with transformed \textit{ORF3} and \textit{ORF5} (BL3+5+) producing fertile gametes. RNA sequencing of tissues collected before (MMC), during (MEI), and after (AME) meiosis of the megaspore mother cell detected 19,269 to 20,928 genes as expressed. Comparison between BL5+ and BL showed that \textit{ORF5} induced differential expression of 6,339, 6,278, and 530 genes at MMC, MEI, and AME, respectively. At MMC, large-scale differential expression of cell wall-modifying genes and biotic and abiotic response genes indicated that cell wall integrity damage induced severe biotic and abiotic stresses. The processes continued to MEI and induced endoplasmic reticulum (ER) stress as indicated by differential expression of ER stress-responsive genes, leading to programmed cell death at MEI and AME, resulting in abortive female gametes. In the BL3+5+/BL comparison, 3,986, 749, and 370 genes were differentially expressed at MMC, MEI, and AME, respectively. Large numbers of cell wall modification and biotic and abiotic response genes were also induced at MMC but largely suppressed at MEI without inducing ER stress and programed cell death, producing fertile gametes. These results have general implications for the understanding of biological processes underlying reproductive barriers.

Reproductive isolation, a phenomenon widely existing in natural organisms, promotes speciation and maintains the integrity of species over time (Coyne and Orr, 2004). It is divided into two general categories depending on whether it occurs before or after fertilization: prezygotic reproductive isolation and postzygotic reproductive isolation (Seehausen et al., 2014). The former prevents the formation of hybrid zygotes, while the latter results in hybrid incompatibility, including hybrid necrosis, weakness, sterility, and lethality in F1 or later generations (Ouyang and Zhang, 2013). The Dobzhansky-Muller model suggests that hybrid incompatibility is caused by the negative interactions between functionally divergent genes in the parental species (Dobzhansky, 1937; Muller, 1942).

\textit{Oryza sativa}, a major food crop and model plant for genomic study of monocotyledon species, also provides a model system for studying reproductive isolation. The Asian cultivated rice is composed of two subspecies, \textit{indica} and \textit{japonica}. Their hybrids are usually highly sterile, which is a barrier for utilization of the strong vigor in \textit{indica-japonica} hybrids. A number of loci responsible for hybrid sterility have been identified, including ones that cause embryo-sac sterility, pollen sterility, and spikelet sterility (Ouyang et al., 2009). So far, six loci responsible for hybrid incompatibility have been cloned. Among them, \textit{DPL1/DPL2} (Mizuta et al., 2010), \textit{S27}/\textit{S28} (Yamagata et al., 2010), and \textit{Sa} (Long et al., 2008) are responsible for pollen fertility, while \textit{hsla}1 (Kubo et al., 2016), \textit{S7} (Yu et al., 2016), and \textit{S5} (Chen et al., 2008; Yang et al., 2012) control female fertility; all of them cause spikelet sterility. Three evolutionary genetic models have been proposed to summarize the findings: parallel divergence, sequential divergence, and parallel-sequential divergence (Ouyang and Zhang, 2013). \textit{DPL1/DPL2} and \textit{S27}/\textit{S28} in rice, \textit{HPA1}/\textit{HPA2} in Arabidopsis (\textit{Arabidopsis thaliana}), and \textit{JaYalpha} in \textit{Drosophila melanogaster} conform to the parallel divergence model. \textit{Sa}, \textit{hsla}1, and many loci in other species can be explained by the sequential divergence model. However, \textit{S5} in rice is the only case identified so far to follow the parallel-sequential divergence model. \textit{S5} is a major locus controlling \textit{indica-japonica} hybrid sterility identified in many studies (Ikehashi and Araki, 2017).

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genes, ORF3, ORF4, and ORF5, in which ORF5+ and ORF4+ together function as the killer of the female gametes and ORF3+ protects the gametes from killing. A typical indica variety has the haplotype of ORF3+, ORF4−, and ORF5+, and japonica variety is of ORF3−, ORF4+, and ORF5−. Balilla (abbreviated BL) is a typical japonica variety. Transgenic plants Balilla ORF5+ (abbreviated BL5+) carrying a transformed ORF5+ under its native promoter showed extremely low spikelet fertility (6.50%) compared with wild-type BL (75.52%). In contrast, Balilla ORF5+ (abbreviated BL3+5+) carrying both transformed ORF5+ and transformed ORF3+ (also under its native promoter), had normal spikelet fertility (77.35%) due to the protective role of ORF3+ (Supplemental Fig. S1, A–C). Paraffin section results showed that the ovule development process in both BL and BL3+5+ was normal. In brief, the archesporial cell enlarged initially and developed into a megaspore mother cell (Fig. 1, A and K). The megaspore mother cell then underwent meiotic division, forming four megaspores in a line (Fig. 1, B, C, L, and M). The three megaspores near the micropyyle degenerated, while the megaspore nearest the chalaza enlarged and developed into a functional megaspore (FM; Fig. 1, D and N). The FM underwent three rounds of mitotic division to produce an eight-nucleate embryo sac (Fig. 1, E and O). In the aborted ovule of BL5+, the megaspore mother cell, dyad, and tetrads could be produced as in BL (Fig. 1, F–H). However, the nucellus cells showed vacuolization and even degeneration at the meiosis stage (Fig. 1, G and H). After meiosis, morphological abnormalities of both nucellus cells and FM were observed (Fig. 11). The abnormal FM did not enlarge and failed to develop into a mature embryo sac. The aborted embryo-sac accumulated unknown substances that were deeply stained (Fig. 1J). Thus, the megaspore meiosis process in BL5+ was normal, but the nucellus cells around the megaspores showed serious defects at the megaspore meiosis stage. The nucellus cell degradation and FM abortion caused the sterility of BL5+.

ORF5 and ORF4 were neighboring genes overlapping in their promoter region (Yang et al., 2012). These kinds of gene pairs are frequently coexpressed (Wang et al., 2009; Kourmpetli et al., 2013). ORF5 and ORF4 had a similar expression profile and relatively high expression in reproductive organs (Yang et al., 2012). The in situ hybridization showed that ORF5+ had localized expression in developing ovules (Chen et al., 2008). Similarly, ORF4+ also displayed high and specific expression in ovule tissues (Supplemental Fig. S2, G–K). The specific expression of ORF5+ and ORF4+ in ovules was in accordance with the specific abortion of ovules in BL5+. However, the protector ORF3+ showed a wide range of expression in many tissues including ovules, indicating its possible function in many processes (Supplemental Fig. S2, A–F).

Based on the ovule development process, we divided the ovule abortion process into three stages (Fig. 1). The first was the megaspore mother cell (MMC) stage when the ovule had megasporocyte and no obvious abnormalities were observed in BL5+. The second was the

RESULTS

Ovule Abortion Process of BL5+

Previous study (Yang et al., 2012) showed that the S5 reproductive barrier is composed of three tightly linked
meiosis (MEI) stage when meiosis was in progress and the nucellus cells of BL5+ were vacuolated. The third was after meiosis (AME) when some unknown substances accumulated in embryo-sac (Fig. 1).

**Processes Underlying Gamete Abortion in BL5+**

We collected the pistils of wild-type BL, sterile BL5+, and fertility-restored BL3+5+ at MMC, MEI, and AME, and performed transcriptomic analysis. A data matrix of three stages from three genotypes was generated. A total of 19,269 to 20,928 genes were detected as expressed in these tissues at different stages representing 34.53 to 37.50% of total annotated genes of the Nipponbare reference genome (Supplemental Table S1; Kawahara et al., 2013). A total of 8,339, 6,278, and 530 genes were differentially expressed in BL5+ compared with BL at MMC, MEI, and AME, respectively, including 4,234, 2,430, and 410 genes that were up-regulated at the three stages and 4,105, 3,848, and 120 genes that were down-regulated (Supplemental Table S2; Supplemental Data Set 1). Among the differentially expressed genes (DEGs), 2,067 were up-regulated and 3,325 were down-regulated at both MMC and MEI (Fig. 2A). These 5,392 common DEGs accounted for 64.66 and 85.89% of total DEGs at MMC and MEI, respectively, indicating that MMC and MEI involved substantially overlapping cellular and molecular responses.

**An Overview of the Differentially Induced Processes in BL5+ Revealed by the Transcriptomes**

We performed MapMan and Gene Ontology (GO) enrichment analyses to classify the DEGs. MapMan is an effective tool to analyze metabolic pathways and other biological processes of the transcriptome systematically (Thimm et al., 2004; Ramsak et al., 2014). To get a global view of the 8,339 DEGs in BL5+ at MMC, we used the “Metabolism overview” and “Cellular response” of MapMan to outline the transcriptional changes of genes with putative functions in metabolism and cellular response (Fig. 2, B and C).

In “Metabolism overview,” primary metabolic processes, including cell wall (354 genes), amino acid (226 genes), lipids (316 genes), nucleotide (81 genes), and major carbohydrate (128 genes) metabolism, accounted for 13.25% of total DEGs. The cell wall localization of ORF5+, which was previously hypothesized to initiate the premature PCD (Yang et al., 2012), led us to focus our attention on the cell wall metabolic genes. We found that 187 (128 up-regulated and 59 down-regulated) of the 354 genes were involved in cell wall degradation and modification, which may affect the cell wall structures in one way or another. The secondary metabolism processes, including terpenes (64 genes), flavonoids (105 genes), and phenylpropanoids (120 genes) metabolism, which are important in plant stress response (Dixon and Paiva, 1995; Winkel-Shirley, 2002; Tholl,

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2006), constituted 3.47% of all DEGs. In the term of “Cellular response,” 8.65% (721 genes) of DEGs were responsive to biotic and abiotic stress; 1.94% (162 genes) of DEGs were related to redox, a phenomenon usually triggered by stress; and 6.67% (667 genes) of DEGs functioned in developmental processes.

GO analysis for up-regulated genes revealed that three GO terms related to cell wall metabolism, seven GO terms involved in stress or stimulus response, and eleven GO terms associated with gene expression, protein synthesis, and translation were significantly enriched (false discovery rate [FDR] < 0.005; Fig. 3).

The change in cell wall structure by cell wall modification genes may cause cell wall integrity damage, which was similar to the situation of pathogen or pest attack. We thus explored the “Biotic stress” in BL5+ at MMC and found that DEGs in various functional categories of biotic stress were enriched, including R genes, pathogen-related proteins, signaling, hormone signaling, proteolysis, ROS regulation, and biotic stress-responsive transcription factors (Supplemental Fig. S3A).

Thus, both the MapMan and GO results indicated that a severe stress response including both biotic and abiotic stresses was induced in BL5+ at MMC, which may be caused by the damage in cell wall integrity, even though no abnormalities were observed at this stage (Fig. 1F).

At MEI, 163 of the 300 DEGs in the “cell wall” were related to the cell wall degradation and modification.

Figure 2. Analysis of genes differentially expressed in BL5+ compared to BL. A, Numbers of the up- and down-regulated DEGs at MMC and MEI. B, Numbers of DEGs in different MapMan functional categories at MMC, MEI, and AME, respectively. C, The heat map of “Metabolism overview” and “Cellular response” in BL5+ at MMC based on the MapMan results. Each small square represents a gene differentially expressed in BL5+ versus BL. The color of the square represents the level of up-regulation (red) or down-regulation (green) based on the log2 fold change of the DEGs.
including 102 up-regulated and 61 down-regulated DEGs (Fig. 2, B and C). Cell wall-related GO terms such as “glucan metabolic process,” “polysaccharide metabolic process,” and “polysaccharide biosynthetic process” were enriched (Fig. 3). The continuous up-regulation of cell wall-degrading and -modifying genes at MMC and MEI might be related to the observed cell wall abnormalities such as vacuolization and degradation at MEI (Fig. 1, G and H). In addition, 599 DEGs (9.54% of total DEGs) in “biotic stress” and “abiotic stress” and genes in various functional categories of biotic stress were identified (Fig. 2B; Supplemental Fig. S3B), suggesting that the stress response also continued at MEI.

By the AME stage, DEG numbers in all the metabolic processes and cellular responses were largely reduced compared to those at MMC and MEI (Fig. 2B). The ovule had aborted with the accumulation of unknown substances. In contrast, however, a GO term “cellulose biosynthetic process” was significantly enriched (Fig. 3), indicating misregulation of cellulose biosynthesis in BL5+.

Large-Scale Transcriptional Changes of Cell Wall-Modifying and -Degrading Genes in BL5+ at MMC and MEI

The MapMan and GO results showed that cell wall metabolism, especially cell wall modification and degradation, were active in BL5+ (Fig. 2). Cell wall-modifying and -degrading genes, also called cell wall-remodeling genes, affect cell growth by changing the extensibility and physiochemical properties of the cell wall (Cosgrove, 1999; ZhiMing et al., 2011; Gou et al., 2012; Hepler et al., 2013; Xiao et al., 2014). We analyzed the expression of genes from seven main enriched families: expansins (EXPs), xyloglucan endotransglucosylase/hydrolases (XTHs), pectin methyl esterases (PMEs), pectin acetyl-esterases (PAEs), polygalacturonases (PGases), glycoside hydrolase family 9 (GH9), and arabinoxylan proteins (AGPs), which affect cell wall structure and extension by enzymatic or nonenzymatic ways (Bosch et al., 2005; Coimbra et al., 2008; Maris et al., 2009; Gou et al., 2012; Che et al., 2016). We found that 95, 85, and 23 genes from these families were differentially expressed at MMC, MEI, and AME, respectively, of which 73 genes were differentially expressed at both MMC and MEI, including 59 up-regulated and 14 down-regulated (Fig. 4, A and B). The 59 up-regulated genes consisted of 13 EXPs, 7 XTHs, 5 PMEs, 4 PAEs, 8 PGases, 7 GH9s, and 15 AGPs. *OsEXP4* was elevated in BL5+ at MMC and MEI. In rice, overexpression of *OsEXP4* results in increased cell length, while repression of *OsEXP4* reduces cell length (Choi et al., 2003). Three up-regulated XTHs (*Os06g48160*, *Os03g01800*, and *Os10g39840*) have been characterized to utilize xyloglucan, one of the cell wall components, as substrate (Hara et al., 2014).

*OsGLU1*, a GH9 member that regulates cell elongation in rice (Zhou et al., 2006; Zhang et al., 2012), was found up-regulated as well. The up-regulation of genes involved in cell wall degradation and cell size regulation was in accordance with cell wall abnormalities in BL5+ (Fig. 1, G and H). Thus, we inferred that the up-regulation of the cell wall-remodeling genes induced by ORF5+ in BL5+ led to cell structure change, damaging the cell wall integrity.

Cellulose Deposition in BL5+

In BL5+, unknown substances were accumulated in the aborted ovule at AME (Fig. 1, I and J). The enrichment of the GO term “cellulose biosynthetic process” suggested a misregulation of cellulose biosynthesis and that the deposited substances in the aborted ovule...
might overaccumulate cellulose. To validate this, we used Pontamine fast scarlet 4B and calcofluor white to stain cellulose (Anderson et al., 2010; Thomas et al., 2012), and observed strong fluorescence signals in BL5+ at the site where the substances accumulated, indicating that cellulose was indeed in large quantity (Fig. 5, B and E), which was also supported by the much higher cellulose content in panicles of BL5+ than in BL (Fig. 5G). In contrast, normal mature embryo-sac structures were easily identified in the ovule of BL and BL3+5+, where low fluorescence intensity was detected (Fig. 5A, C, D, and F).

Cellulose is a major cell wall polysaccharide composed of unbranched β-1, 4-linked glucan chains. In rice, OsMYB46 and OsSWN are secondary cell wall biosynthetic regulators. OsSWN1 acts on the upstream of OsMYB46 by binding to the SNBE element in its promoter region. Overexpression of OsSWN1 or OsMYB46 in Arabidopsis results in the ectopic deposition of cellulose, xylan, and lignin (Zhong et al., 2011). In BL5+, OsSWN1, OsSWN4, OsSWN6, and OsMYB46 were up-regulated at MMC and MEI. Three cellulose synthases, OsCESA4, OsCESA7, and OsCESA9, which are regarded as subunits of cellulose synthase complex (Tanaka et al., 2003), were continuously up-regulated from MMC to AME. In addition, expression of five cellulose synthase-like genes (OsCslA1, OsCslA3, OsCslA5, OsCslA9, and OsCslF6) and two brittle culm genes (BC1 and BC3) were also elevated at MMC or MEI (Fig. 4C). Upstream transcription factors such as OsSWN1, OsSWN6, and OsMYB46 had peak expression at MEI, while their downstream genes such as OsCESAs and two brittle culm genes had the highest expression at AME (Supplemental Fig. S4), in agreement with the overaccumulation of cellulose at AME (Fig. 5, B and E). These results showed that the cellulose biosynthetic pathway was highly activated in BL5+, resulting in the accumulation of cellulose in the aborted embryo-sac.

**Callose Deposition in BL5+**

Callose is a dynamic component of the plant cell wall that plays important roles in meiosporogenesis. The
uneven distribution of the callose wall around the megaspores decides the polarization of megaspores, the formation of a functional megaspore, and the abortion of the other three nonfunctional megaspores (Ünal et al., 2013). Since many genes encoding β-1,3-glucanases, which have putative functions in callose metabolism, were differentially expressed in BL5+ at MMC and MEI (Supplemental Fig. S5), we investigated the dynamic callose distributions in ovule of BL, BL5+, and BL3+5+. During meiosis, callose preferentially accumulated at the micropylar end of megaspores. Two callose bands were observed in all three genotypes at the dyad stage (Fig. 6, A–F), and at the tetrad stage at least three callose bands could be recognized also in all three genotypes although slightly different in distribution patterns (Fig. 6, G–L). This apparent normal callose distribution in megaspores of BL5+ indicated likely normal meiosis. Unlike BL and BL3+5+, however, weak callose deposition was detected in nucellus cells at the tetrad stage in BL5+ (Fig. 6I). At the functional megaspore (FM) stage, callose accumulated in the three degenerated gametes but not in the FM (Fig. 6, M–R). The survived FM subsequently developed to embryo-sac (Fig. 6, S, T, W, and X). However, the ovule of BL5+ displayed irregular callose deposition around the FM and in nucellus cells (Fig. 6, O and P), and finally, the aborted embryo-sac was stuffed with a large amount of callose (Fig. 6, U and V). Thus, ORF5+ resulted in a misregulation of callose deposition during megasporogenesis such that callose began to accumulate abnormally from MEI and was deposited in a large quantity at AME in the ovule of BL5+.

**ER Stress and Premature PCD Induced in BL5+**

Under stress conditions, the number of misfolded proteins will increase and may exceed the capacity of the ER quality control system, thus inducing ER stress (Howell, 2013; Wan and Jiang, 2016). In BL5+, 78.95% of genes in “RNA transcription,” 66.23% of genes in “RNA processing,” 57.69% of genes in “protein synthesis initiation,” 58.33% of genes in “protein synthesis...
elongation,” 82.46% of genes in “ribosomal protein synthesis,” and 60.91% of genes in “ribosome biogenesis” were up-regulated at MMC (Supplemental Fig. S6), suggesting a greatly increased level of protein synthesis, which may increase the protein folding burden on ER. To detect whether ER stress occurred in BL5+ at MMC, we examined the expression of ER stress-responsive genes in BL5+ and found that 10 such genes were significantly up-regulated, including OsbZIP50, which is a key ER stress regulator in rice (Fig. 7A). The mRNA of OsbZIP50 was spliced when ER stress occurred (Hayashi et al., 2012). We then detected the unconventional splicing of OsbZIP50 mRNA in different materials and found that OsbZIP50 mRNA was spliced in BL5+ at MMC, but not in BL or BL3+5+ (Fig. 7B). The spliced OsbZIP50 mRNA encodes a transcription factor OsbZIP50-S, which binds to the pUPRE-II cis-element in the promoter regions of OsBIP4, ORF3, OsEBS, and OsPDIL2-3 (Hayashi et al., 2013; Takahashi et al., 2014; Wakasa et al., 2014). Accordingly, expression of these four genes was elevated (Fig. 7A). Some other genes including Fes1-like, CRT2-2, CRT1, and SAR1B-like, which were induced in ER stress, were also up-regulated. These results showed that ER stress occurred in BL5+ at MMC relative to BL and BL3+5+. In response to ER stress, genes involved in protein modification (303 up-regulated and 358 down-regulated) and targeting (70 up-regulated and 53 down-regulated) were differentially expressed, presumably to help protein processing and transportation. In addition, 793 DEGs (339 up-regulated and 454 down-regulated) involved in “protein degradation” were found, likely as a remedy to degrade the misfolded or unfolded proteins (Supplemental Fig. S6). The splicing of OsbZIP50 mRNA and up-regulation of ER stress-responsive genes were also detected at MEI and AME (Fig. 7, A and B), indicating that ER stress proceeded continuously in BL5+.

The unresolved ER stress could induce premature programmed cell death (PCD) (Cai et al., 2014). In BL5+, PCD signals were detected from the meiosis stage (Yang et al., 2012), corresponding to the MEI stage in this study. At MEI, we detected up-regulation of two aspartic protease-encoding genes, OsAP25 and OsAP37 (Fig. 7, A and C), both of which participate in the tapetal PCD process and induce PCD in yeast and plants (Niu et al., 2013).

**Figure 7.** Differential regulation of ER stress and PCD genes. A, Log2 fold change of the expression level of ER stress and PCD genes in BL5+ against BL. The color in each cell represents the levels of expression change based on the log2 fold. The cell without color indicates the gene was not differentially expressed. B, Splicing of OsbZIP50 mRNA in BL, BL5+, and BL3+5+ at each stage. C, qPCR verification for expression of tapetal PCD-related genes in BL and BL5+. The x axis represents different stages. The y axis represents the expression level relative to profilin (Os06g05880). The data are means ± se (n = 3).
Overexpression of OsAP25 or OsAP37 in plants results in cell death, which is suppressed by aspartic protease-specific inhibitor pepstatin A. Transformation of OsAP25 or OsAP37 into a PCD-deficient yeast strain restores the PCD ability of the strain. OsAP25 showed the highest expression at MEI (Fig. 7C), consistent with the observation of PCD signals at this stage. In the tapetal PCD process, transcription factor EAT1 directly regulates the expression of OsAP25 and OsAP37 (Niu et al., 2013). However, EAT1 was down-regulated at MEI, indicating that some other regulators might have replaced EAT1 to activate OsAP25 and OsAP37. TDR and TIP2, which work upstream of OsAP25 and OsAP37 in pollen development (Niu et al., 2013), were up-regulated in BL5+ at MMC and MEI (Fig. 7, A and D). The coordinated up-regulation of TDR, TIP2, OsAP25, and OsAP37 suggested that the ovule and tapetum may share some common genes/pathways in PCD. Taken together, an OsbZIP50-dependent ER stress pathway and a premature PCD pathway involving the tapetal PCD genes were activated in BL5+.

These results suggested that the ORF5+-induced up-regulation of cell wall degradation and modification genes in BL5+ caused cell wall damage, which induced both biotic and abiotic stresses. Lasting stress responses induced ER stress and unresolved ER stress led to premature PCD, eventually resulting in the ovule abortion.

**Processes Detected in the BL3+5+/BL Comparison**

BL3+5+ carrying both the killer ORF5+ and the protector ORF3+ showed no phenotypic difference from the wild-type BL with respect to fertility (Supplemental Fig. S1). However, 3,986 DEGs were still identified between BL3+5+/BL and BL5+/BL comparisons at different stages. The overlapping areas indicate the genes differentially expressed in both BL3+5+ and BL5+. A, The number of DEGs in different functional categories of “Metabolism overview” and “Cellular response” by MapMan at MMC, MEI, and AME, respectively. C, Partial significantly (FDR < 0.005) enriched GO terms for up-regulated genes in BL3+5+ compared with BL. The color in each cell represents the significance of enrichment based on the FDR value. The cells without color indicate not significantly enriched. The full list of GO terms is given in Supplemental Table S4.
were found (Fig. 4, A and B), suggesting that cell wall integrity damage was suppressed. The cell wall compensation pathways such as cellulose biosynthesis and callose deposition were not activated (Figs. 4C and 6, K and Q), and ER stress and PCD did not occur (Fig. 7). As a result, the cell wall maintained integrity and the ovule meiosis was undisturbed (Fig. 1, L and M).

At AME, the number of DEGs was further reduced to 370 in BL3+5+ compared with BL (Supplemental Table S2; Supplemental Data Set 2). These results indicated that ORF5+ induced similar but weaker damage processes in BL3+5+ as in BL5+ at MMC, but these processes were soon suppressed by ORF3+. Consequently, the embryo-sac developed normally, producing fertile female gametes (Fig. 1O).

**DISCUSSION**

The killer-protector system of S5 is composed of three proteins: cell wall protein ORF5+, transmembrane protein ORF4+, and ER resident protein ORF3+. Based on the comparative transcriptome results of BL5+ and BL3+5+, we proposed a model to summarize the processes underlying the S5 system (Fig. 9).

In BL5+, many cell wall-modifying and -degrading genes in the families EXPs, XTHs, PMEs, PAEs, PGases, GH9, and AGPs were up-regulated at MMC and MEI, and many genes of these families play important roles in cell elongation or cell expansion (Zhou et al., 2006; Chen and Ye, 2007; Yang et al., 2007; Coimbra et al., 2008; Maris et al., 2009; Zhang et al., 2010, 2012; Zhu et al., 2011; Gou et al., 2012; Ma et al., 2013; Miedes et al., 2013; Harai et al., 2014; Xiao et al., 2014; Che et al., 2016). It has been suggested that postsynthetic modifications of the cell wall by cell wall-modifying genes may affect cell wall integrity (CWI; Pogorelko et al., 2013). Thus, large-scale up-regulation of cell wall-remodeling genes induced by ORF5+ may lead to changes of cell wall structures, which affect CWI, thus inducing stress response.

ORF5+ encodes an extracellular aspartic protease. In *Pichia pastoris* and *Saccharomyces cerevisiae*, extracellular aspartic protease yapsins are required for the CWI maintenance (Krysan et al., 2005; Guan et al., 2012). Two plant aspartic proteases, A36 and A39, participate in cell wall remodeling in pollen tube (Gao et al., 2017). The study in *Candida albicans* shows that secreted aspartic proteinases use a set of cell wall proteins as substrates (Schild et al., 2011). We speculated that one or more substrates of ORF5+ existed in the rice cell wall, which induced cell wall damage after activation by ORF5+. In *Saccharomyces cerevisiae*, CWI damage triggers unfolded protein response, a homeostatic response to alleviate ER stress, which in turn affects the CWI (Krysan, 2009; Scrimale et al., 2009). In plants, pathogen invasion or pest attack can induce CWI damage and subsequent stress response (Bellincampi et al., 2014; Pogorelko et al., 2013). CWI damage eventually leads to PCD (Paparella et al., 2015). In BL5+, the functional categories responsive to pathogen invasion or pest attack were enriched, and ER stress was accordingly suppressed, which induced cell wall damage after activation by ORF5+.

Figure 9. A model for S5 locus-mediated ovule abortion.

BL3+5+ and BL at MMC (Supplemental Table S2; Supplemental Data Set 2), of which 3,843 DEGs were shared with the BL5+/BL comparison at this stage, indicating that ORF5+ induced similar processes in BL3+5+ (Fig. 8A). As expected, the process of “cell wall” was enriched with 217 DEGs (Fig. 8B), of which 64 (55 up-regulated and 9 down-regulated) were cell wall-modifying and -degrading genes from the seven main families (Fig. 4, A and B). GO terms “glucan metabolic process” and “polysaccharide metabolic process” were significantly enriched in up-regulated genes (Fig. 8C). Terms “biotic stress” and “abiotic stress” were also enriched involving 339 DEGs. However, the number of DEGs in each of the enriched functional categories was smaller than that in the BL5+/BL comparison (Fig. 4, A and B). Moreover, the splicing of OsbZIP50 mRNA and the up-regulation of its downstream genes were not detected (Fig. 7, A and B). These results suggested that ORF5+ induced similar processes in BL3+5+ at MMC, especially with respect to cell wall integrity damage, but to a much lesser extent, which did not trigger ER stress.

At MEI, the difference between BL3+5+ and BL was greatly narrowed such that only 749 DEGs were identified, compared with 6,278 genes in the BL5+/BL comparison (Supplemental Table S2; Supplemental Data Set 2). Thus, numbers of DEGs, especially those in cell wall, biotic stress, and abiotic stress, were greatly reduced (Fig. 8B). Only seven DEGs from the seven cell wall families were found (Fig. 4, A and B), suggesting that cell wall integrity damage was suppressed. The cell wall compensation pathways such as cellulose biosynthesis and callose deposition were not activated (Figs. 4C and 6, K and Q), and ER stress and PCD did not occur (Fig. 7). As a result, the cell wall maintained integrity and the ovule meiosis was undisturbed (Fig. 1, L and M).
induced, presumably by CWI impairment, which in turn exacerbated the cell wall damage and its subsequent stress responses, thus finally leading to PCD. To alleviate the adverse effects caused by CWI damage, cells usually trigger a cell wall compensatory pathway to strengthen the cell wall (Hamann, 2012). For example, disruption by aspartic protease Yps7p in Pichia pastoris reduces chitin content in the lateral cell wall, but the cell thickens the inner cell wall for compensation (Guan et al., 2012). Similarly, in BL5+, key genes in secondary cell wall biosynthesis were significantly up-regulated, resulting in the cellulose accumulation to repair cell wall. Callose, a polysaccharide usually accumulated under stress conditions to provide physical barrier to the adverse environments (Pirselova and Matusikova, 2012), was deposited in large quantity. Despite other roles of callose in ovule development, the large amount deposition of callose in BL5+ was most likely to strengthen cell wall.

ORF4+ acted as a partner of ORF5+ with a transmembrane localization. We speculated that ORF4+ functioned as a sensor of CWI damage. In plants, a well-studied cell wall sensor is ATWAK1, a wall-associated kinase, which resides in the plasma membrane and responds to oligogalacturonides derived from the hydrolysis of cell wall component (Brutus et al., 2010). Xad4, a wall-associated kinase in rice, contributes to disease resistance by strengthening the cell wall via promoting cellulose synthesis and suppressing cell wall loosening (Hu et al., 2017). Unlike wall-associated kinase, no extracellular binding domain or inner kinase domain was found in ORF4+; thus there may exist a coreceptor that interacts with ORF4+ in the plasma membrane and/or a downstream signal protein that interacts with plasma membrane receptor to transmit signals.

Based on these analyses, it was deduced that ORF5+ damaged CWI by cleaving its substrates, leading to up-regulation of the cell wall-modifying and -degrading genes. ORF4+ sensed the CWI impairment and induced biotic and abiotic stress responses, and consequently induced ER stress due to the accumulation of drastically increased misfolded proteins in ER. The occurrence of ER stress in turn deteriorated the CWI damage and led to a more serious stress response. Finally, the unresolved cell wall damage, stress response, and ER stress triggered PCD. Meanwhile, the cellulose and callose accumulated in the ovule as the cells’ unsuccessful remedy to repair the damaged cell wall.

BL3+5+ showed no difference with BL with respect to ovule development and fertility, but a number of cell wall-modifying and -degrading genes were still up-regulated in BL3+5+ at MMC, which may induce CWI damage. However, transcriptional change of these genes was suppressed at MEI and AME. Thus, the subsequent negative effects caused by CWI damage, including ER stress, PCD, cellulose, and callose deposition, were prevented in BL3+5+. ORF3+ encodes a HSP 70 chaperone, which is a downstream target of OsbZIP50s and induced by ER stress. ORF3+ is believed to help protein folding in ER to resolve ER stress like OsBIP1-OsBIP5 in the HSP70 gene family (Yang et al., 2012; Hayashi et al., 2013; Wakahara et al., 2014). BIP protein alleviates Cd2+ stress-induced PCD (Xu et al., 2013). The tobacco (Nicotiana tabacum) plants with overexpression of BIP show delayed leaf senescence (Carvalho et al., 2014). In BL3+5+, ORF3+ suppressed the ORF5+-induced ER stress and PCD. It was speculated that one role of ORF3+ was alleviating ER stress thus not to induce premature PCD. The chaperone activity of ORF3+ may also help the folding of the signal proteins downstream of ORF5+ and ORF4+ so that the stress response may be suppressed at MEI.

In conclusion, we revealed the processes underlying a reproductive barrier composed of ORF3, ORF4, and ORF5 at the transcriptomic level and proposed a mechanistic model based on the results. Nevertheless, further studies are needed to support and enrich the model.

MATERIALS AND METHODS

Sample Preparation and Library Construction

Balilla was a typical japonica rice (Oryza sativa) variety. The transgenic plant BalillaORF5+ was generated by introducing ORF5+ from an indica variety Nanjing 11 into Balilla under its native promoter (Chen et al., 2008). The plant Balilla/ORF3+ORF5+ was isolated from the hybrid offspring between Balilla/ORF5+ and Balilla/ORF3+, which contained a transformed ORF3+ from Nanjing 11 under its native promoter (Yang et al., 2012). All the rice plants were grown in the fields of the experimental station of Huazhong Agricultural University, Wuhan, China. We determined the relationship between the floret length and stage of ovule development by the paraffin section observation of florets from the variety Balilla. The pollen failed to florets of Balilla, Balilla/ORF3+, and Balilla/ORF3+ORF5+ ranging in length from 3.8 to 4.2 mm, 5.5 to 6.0 mm, and 7.0 to 7.5 mm, respectively, were collected as MMC, MEI, and AME stage samples with two biological replicates. RNA was extracted using TRIZol reagent (Invitrogen) according to the manufacturer’s manual. RNA yields and quality were measured using Nanodrop 2000 (Thermo Scientific) and further quantified using Qubit2.0 fluorometer (Invitrogen). RNA integrity was evaluated using Experion RNA Analysis Kits (Bio-Rad) and Experion Automated Electrophoresis Station (Bio-Rad). Each library was constructed using 3 μg total RNA using the TruSeq RNA Library Preparation Kit v2 (Illumina). The libraries were sequenced using Illumina HiSEquation 2000 sequencing platform at National Key Laboratory for Crop Genetic Improvement in Huazhong Agricultural University, Wuhan, China.

Data Processing and DEG Identification

The data of the two replicates of all samples were collected and analyzed following the workflow described previously (Trapnell et al., 2012). In brief, the raw sequencing reads were mapped to the rice reference genome (MSU7.0, http://rice.plantbiology.msu.edu/index.shtml) using TopHat, then the transcripts were assembled and quantified using Cufflinks, and finally, differentially expressed genes were analyzed using Cuffdiff. The raw data files can be found in the NCBI database under the accession number GSE95200. Genes with ≥2-fold expression up-regulation or down-regulation and adjusted P value ≤ 0.05 (counted using Cuffdiff) were regarded as up-regulated or down-regulated genes.

GO and MapMan Analysis

GO enrichment was performed in AgriGO, a web-based tool for GO analysis (Du et al., 2010; http://bioinfo.cau.edu.cn/agriGO/index.php). The species “Oryza sativa” was chosen. GO terms in biological process groups with FDR < 0.05 were identified and analyzed further. The MapMan software version 3.5.1.R2 and pathways were downloaded from the MapMen Site of analysis (http://mapman.gabipd.org/web/guest/mapmanstore). The mapping information of rice genes (osa_MSU_v7.2015-09-08_mapping.txt.tar.gz) that can be imported to the MapMan software was downloaded from GoMapMan (http://www.gomapman.org/export/current/mapman; Ramsak et al., 2014).
**In Situ Hybridization**

The methods for preparation of paraffin embedded sections followed the previously described protocol (Weng et al., 2014). Primer pairs (listed in Supplemental Table S4) ORF3insituF and ORF3insituR, ORF4insituF and ORF4insituR were used to prepare probes for ORF3 and ORF4, respectively. Products amplified from ORF3+ or ORF4+ cDNA were ligated to pgEM-T vector (Promega). The sense probe was transcribed with T7 RNA polymerase, and the antisense probe was transcribed with SphI RNA polymerase. The probes were labeled with digoxigenin (Roche). Hybridization and detection were performed as described previously (De Block and Debrouwer, 1993).

**PCR and RT-qPCR**

Total RNA of pistils was isolated with TRIzol reagent (Invitrogen) and treated with RNase-free DNase (Invitrogen) for 20 min at room temperature to digest the contaminating DNA. The first-strand cDNA was synthesized using Superscript reverse transcriptase (Invitrogen) with Oligo(dT)15 Primer (Promega) following the manufacturers' instructions. For amplification of OsZIP50b, specific primers around the splice site were used. PCR conditions and PCR product detection were performed as described before (Yang et al., 2012). For quantitative real-time PCR, we carried out the reaction in a total volume of 5 μL containing 5 μL FastStart Universal SYBR Green Master (Rox; Roche), 1 μL cDNA sample, and 3 μL each primer on ABI 7500 system. The primers for PCR and RT-qPCR are listed in Supplemental Table S4. The gene profilin (Os06g05880) was used as the internal control in RT-qPCR (Yang et al., 2012).

**Histological Staining**

The fresh florets were fixed with FAA solution (50% ethanol, 5% acetic acid, and 3.7% formaldehyde) at 4°C overnight, dehydrated with ethanol in a series of concentrations (50, 70, 85, 95, and 100%), infiltrated with xylene, and finally embedded in paraffin. The materials were cut into 10-μm-thick sections and then washed in a xylene/ethanol series (0–50% ethanol). For cellULO staining, the rehydrated sections were stained with 0.1% aniline blue solution for 60 min. For cellulose staining, the rehydrated sections were stained with 0.01% Pontamine Fast Scarlet 4B solution for 20 min or 0.1% calcofluor white (Sigma-Aldrich). The slides were scanned under light microscopy.

**Cellulose Content Determination**

The cellulose content was determined as described previously (Guo et al., 2014). In brief, the materials were suspended with potassium phosphate buffer (pH 7.0), 3.7% formaldehyde (1:1, v/v), dimethyl sulfoxide-water (9:1, v/v), 0.5% (w/v) ammonium oxalate, and 4 μM K2HPO4 containing 1.0 mg/ml sodium borohydride in turn. The remaining pellet was collected and extracted further with H2SO4 (67%, v/v), and the supernatants were collected for the determination of cellulose.

**Accession Numbers**

Sequence data from this article can be found in the GenBank libraries under accession numbers JX384991 (ORF3+), JX384981 (ORF3−), JX385021 (ORF4+), JX385031 (ORF4−), EU889295 (ORF5+), and EU889294 (ORF5−).

**Supplemental Data**

The following supplemental materials are available.

- **Supplemental Figure S1.** The fertility of BL, BL5+, and BL3+5+.
- **Supplemental Figure S2.** RNA in situ hybridization of ORF3+ and ORF4+.
- **Supplemental Figure S3.** DEGs in “Biotic stress” in BL5+ compared with BL at MMC and MEI.
- **Supplemental Figure S4.** qPCR verification for expression of the cellulose biosynthetic genes in pistils of BL, BL5+, and BL3+5+ at MMC, MEI, and AME, respectively.
- **Supplemental Figure S5.** Expression change of genes involved in cellulose metabolism.
- **Supplemental Figure S6.** The number of DEGs involved in RNA transcriptions, protein synthesis, and processing.
- **Supplemental Table S1.** The number of expressed genes in BL, BL5+, and BL3+5+ at different stages.
- **Supplemental Table S2.** The number of differentially expressed genes identified in different comparisons.
- **Supplemental Table S3.** Full list of significantly enriched GO terms (FDR < 0.005) for DEGs in BL5+ versus BL.
- **Supplemental Table S4.** Full list of significantly enriched GO terms (FDR < 0.005) for DEGs in BL3+5+ versus BL.
- **Supplemental Table S5.** The list of cell wall-modifying or -degrading genes and their expression changes based on log2 fold.
- **Supplemental Table S6.** The primers used in this study.
- **Supplemental Data Set 1.** The differentially expressed genes in BL5+ compared with BL.
- **Supplemental Data Set 2.** The differentially expressed genes in BL3+5+ compared with BL.

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**LITERATURE CITED**


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