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Proteomic and phosphoproteomic analyses reveal extensive phosphorylation of regulatory proteins in developing rice anthers

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SUMMARY

Anther development, particularly around the time of meiosis, is extremely crucial for plant sexual reproduction. Meanwhile, cell-to-cell communication between somatic (especial tapetum) cells and meiocytes are important for both somatic anther development and meiosis. To investigate possible molecular mechanisms modulating protein activities during anther development, we applied high-resolution mass spectrometry-based proteomic and phosphoproteomic analyses for developing rice (*Oryza sativa*) anthers around the time of meiosis (RAM). In total, we identified 4984 proteins and 3203 phosphoproteins with 8973 unique phosphorylation sites (p-sites). Among those detected here, 1544 phosphoproteins are currently absent in the Plant Protein Phosphorylation DataBase (P^3DB), substantially enriching plant phosphorylation information. Mapman enrichment analysis showed that 'DNA repair','transcription regulation' and 'signaling' related proteins were overrepresented in the phosphorylated proteins. Ten genetically identified rice meiotic proteins were detected to be phosphorylated at a total of 25 p-sites; moreover more than 400 meiotically expressed proteins were revealed to be phosphorylated and their phosphorylation sites were precisely assigned. 163 putative secretory proteins, possibly functioning in cell-to-cell communication, are also phosphorylated. Furthermore, we showed that DNA synthesis, RNA splicing and RNA-directed DNA methylation pathways are extensively affected by phosphorylation. In addition, our data support 46 kinase-substrate pairs predicted by the rice Kinase-Protein Interaction Map, with SnRK1 substrates highly enriched. Taken together, our data revealed extensive protein phosphorylation during anther development, suggesting an important post-translational modification affecting protein activity.

Keywords: ***Oryza sativa*, mass spectrometry, phosphoproteomics, meiosis, anther development.**

INTRODUCTION

Rice (*Oryza sativa*) is one of the major crops worldwide, providing food for more than half of the population globally; therefore it is a huge challenge to maintain and increase rice yield for food security in the face of population explosion and climate changes (Zuo and Li, 2013). In addition, rice also serves as an excellent model for monocotyledonous plants to investigate molecular and genetic mechanisms of developmental processes (Zuo and Li, 2013). As the male reproductive organ, the anther produces the progenitor cells for and support meiosis, which generate microspores. The microspores then further develop to male gametophytes (pollen), which produce and ultimately deliver sperm cells to the ovule for the completion of double fertilization in higher plants. Although microarray and next generation DNA sequencing (NGS) technologies have been used to investigate gene expression profiles and regulatory networks in the rice anther (Tang *et al.*, 2010; Bai *et al.*, 2015; Deveshwar *et al.*, 2011), the global protein expression levels and post-translational modification in the rice anther remain largely unknown.

Anther development around the time of meiosis is a pivotal stage in flowering plants. In both dicots and

monocots, each lobe of meiotic stage anther consists of the microsporocytes (pollen mother cells) at the center, surrounded by the tapetum and three other somatic layers: the middle layer, endothecium and epidermis from the interior to the surface (Ma, 2005). The tapetum directly contacts the meiocytes,acting as a nursery cell layer to supply water, nutrients, enzymes and developmental signals for normal meiocytes and gametophyte development (Ma, 2005). Tapetum defects could severely affect male gametophyte formation; for example, a mutation of rice *Tapetum Degeneration Retardation* (*TDR*) gene, which encodes a basic helix-loop-helix (bHLH) transcription factor, caused microspores collapse due to tapetum defects (Li *et al.*, 2006). As sexual reproductive cells, meiocytes undergo meiosis to generate haploid spores (Ma, 2005). In the past decades, molecular and genetic studies have identified a number of rice genes involved in various meiotic processes, including homologous paring, synapsis, meiotic recombination and chromosome segregation (Nonomura *et al.*, 2007; Wang *et al.*, 2010, 2011, 2012a; Luo *et al.*, 2013; Miao *et al.*, 2013). Among these, several kinase genes have been shown to play essential roles in rice meiosis, including *Bub1-RELATED KINASE 1* (*BRK1*) and *Multiple Sporocyte* (*MSP1*) (Nonomura *et al.*, 2003; Wang *et al.*, 2012a). These findings suggested that protein phosphorylation is an important post-translation modification regulating meiotic process in rice. Furthermore, it is known that active cell-to-cell communication between tapetum and meiocyte plays an important role to coordinate development of both types of cells, ensuring normal gametophyte formation. Recently, one study has revealed that the rice Microspore and Tapetum Regulation1 (MTR1), a secretory fasciclin glycoprotein, is secreted by the meiocytes to regulate male reproductive cells and somatic cells development (Tan *et al.*, 2012). Obviously, identification of more factors involved in this process will be helpful for comprehensively understanding its molecular mechanisms.

To investigate gene regulation networks controlling anther development, globe gene expression profiles have been examined using microarray hybridization and/or RNA sequencing analyses in developing rice anthers (Deveshwar *et al.*, 2011). However, the relationship between the levels of message RNAs and the abundance of the protein products is not linear. Moreover, post-translational modification of proteins, the subcellular localization of proteins or protein migration, and protein-protein interactions are not possible to study using mRNA expression information. Therefore, a systematic proteomic study could provide additional valuable information for further understanding the molecular mechanisms controlling anther development at protein and post-translational levels. Protein phosphorylation, which is one of the most abundant and best understood post-translational modifications, plays a crucial role in governing signal transduction pathways by modulating protein activity and protein-protein interactions (Mithoe and Menke, 2011). Phosphorylation has been estimated to affect one-third of the total proteins in eukaryotes (Qeli and Ahrens, 2010), and is regulated by the dynamically balanced actions of protein kinases and protein phosphatases. Phosphoproteomic studies could identify more than 20 000 phosphorylation sites in a single project (Olsen *et al.*, 2010). In eukaryotes, serine (Ser), threonine (Thr) and tyrosine (Tyr) are the most common sites for phosphorylation (Pearlman *et al.*, 2011).

In yeast, various protein kinases are involved in distinct meiotic processes, such as chromosome synapsis, homologous recombination, chromosome segregation and kinetochore formation (Rockmill and Roeder, 1991; Leem and Ogawa, 1992; Clyne *et al.*, 2003; Brar *et al.*, 2006). Phosphorylation of Rec8, a meiosis-specific cohesin subunit and regulated by CDC5, is critical for the stepwise loss of cohesins from chromosomes during meiosis I (Rockmill and Roeder, 1991; Leem and Ogawa, 1992; Clyne *et al.*, 2003; Brar *et al.*, 2006). However, only two potential kinases have been discovered to be involved in rice meiosis so far (Nonomura *et al.*, 2003; Wang *et al.*, 2012a). Although genetic and molecular studies have shown the rice homolog of yeast Rec8, OsREC8, is also essential for meiosis (Shao *et al.*, 2011), its phosphorylation modification has not been reported. Thus, a globe investigation of phosphorylated proteins in rice anther will be not only important for deeper understanding the molecular mechanism of some already known functional proteins, but also crucial for revealing protein-protein interaction networks involved in phosphorylation modification.

Here, we took the advantages of mass spectrometry (MS) to analyze phosphoproteome as well as proteome of rice anthers around the time of meiosis (hereafter referred to as RAM), aiming to provide a protein phosphorylation map for RAMs. Thus, analysis of the obtained data would enable us to directly see the specific phosphorylated sites for a large number of phosphoproteins, and also help us to infer their functional regulation in rice meiosis, tapetum and their coordinated development. In this study, totally, we identified 4984 proteins and 3203 phosphoproteins with 8973 uncovered phosphorylation sites, and further analyzed the data for functions potentially affected by phosphorylation. We believed that the information presented here not only improved our understanding about the role of protein phosphorylation in RAM, but also provided a large number of phosphorylation sites for potentially critical proteins in this process.

RESULTS AND DISCUSSION

Characterization of the RAM proteome and phosphoproteome

Based on the morphological features of rice panicles containing anthers with meiocytes (Cheng, 2013), we collected

RAMs from wild-type rice plants (Figure S1a–c). Histological sections revealed that the obtained materials included stage-5 to 8 anthers as defined by Cheng (Cheng, 2013), from pre-meiotic pollen mother cells to microspores just released from tetrads (Figure S1d–g). In total, we harvested about 10 000 RAMs for further experiments. Subsequently, we employed Nano UHPLC-MS/MS to detect the protein and phosphorylated protein profiles followed by the streamlined approaches (Figure S1 h). In this study, we identified 24 087 peptides, representing 4984 non-redundant individual proteins (Table S1). To investigate the potential correlation between mRNA expression and protein abundance, we compared the microarray data from rice meiotic anthers (Deveshwar *et al.*, 2011) and proteomic data in this study. In total, 1650 genes with expression data in the microarray analysis were detected in our proteome. After performing a scattered plot for mRNA expression value against protein abundance, we found that there is a very slight correlation between them (Figure S2a), consistent with the lack of good correlation also found in prokaryotes and mammals (Nie *et al.*, 2006; Bauernfeind *et al.*, 2015; Swindell *et al.*, 2015).

Meanwhile, our phosphoproteomic analysis detected 9594 unique phosphopeptides, which were assigned to 3203 proteins with 8973 detected phosphorylation sites (Table S2). Among of them, 1847 phosphorylated proteins were exclusively found in the phosphoproteome (Figure 1a), which likely benefited from the improved

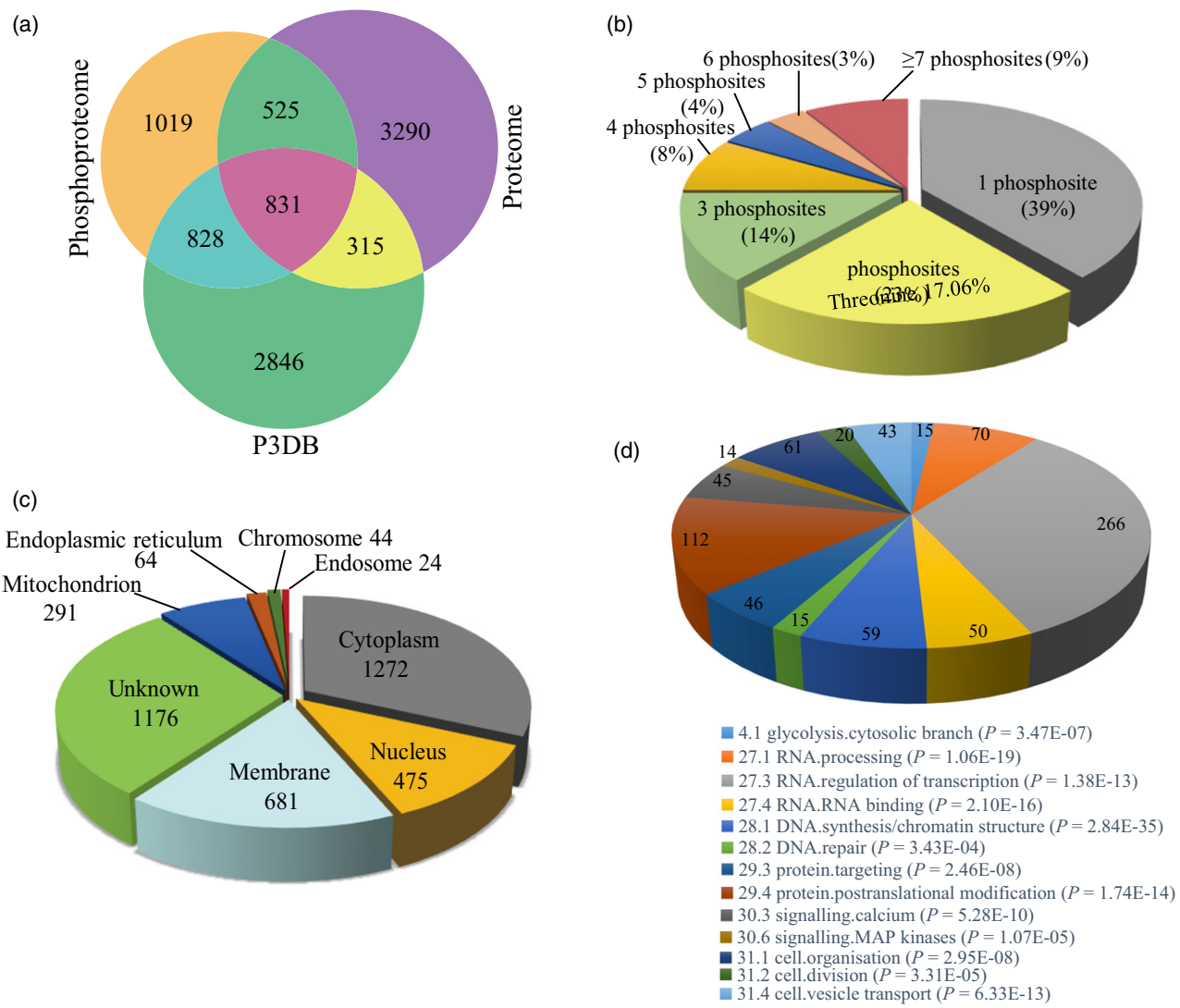

Figure 1. Characteristics of the proteome and phosphoproteome of rice anthers.
(a) Overlap of the anther phosphoproteome with proteome and P^{3}DB datasets.
(b) Proportion of singly and multiply phosphorylation sites per protein in RAM phosphoproteome.
(c) The subcellular localization of rice anther phosphoproteins predicated by GO annotation.
(d) Enriched biological functions (secondary MapMan bins) of rice anther phosphoproteome. Only the bins with $P < 1.0E-02$ are presented.

phosphopeptides enrichment in this study. Thus, combining two datasets, we totally identified 6831 proteins presented in RAMs. A comparison with the Plant Protein Phosphorylation DataBase (P^3DB) (Yao *et al.*, 2014) revealed that 1544 phosphoproteins in our RAMs dataset were absent from the current P^3DB (Figure 1a). Thus, our data not only greatly enlarged the Plant Protein Phosphorylation DataBase, but also paved a way for functionally investigating proteins with phosphorylation modification during rice anther development.

The number of phosphorylation sites in a specific phosphoprotein varies considerably. Although most proteins had only a few detected phosphorylate sites, a number of proteins were identified with more than 20 phosphorylation sites (Table S3). The largest number of phosphorylation sites is 34 for LOC_Os01g04650, which encodes a PB1 domain-containing protein (Table S3). The percentages of phosphoproteins with different numbers of phosphorylation sites are shown in Figure 1(b), with 39% having one detected phosphorylation site, and 23% having two sites. It is worth noting that about 9% (275 proteins) of the identified phosphorylated proteins have over seven phosphorylation sites, including 36 transcription factors (Table S3), suggesting extensive regulation of the transcriptional networks by protein phosphorylation. As for the phosphorylated amino acid residues, among the 8973 identified phosphorylation sites, 81.33% (7236) are phosphorylated on serine (pSer), 17.06% (1518) on threonine (pThr) and 1.6% (143) on tyrosine (pTyr) (Figure S2b), very similar to the previously reported results in eukaryotes (Myers *et al.*, 2012).

Although the frequency of Tyr phosphorylation is very low, a few cases have been found in plants, especially in reproductive tissues. For instance, a phosphoproteomic study of Arabidopsis mature pollen detected one pTyr-containing peptide (Mayank *et al.*, 2012). Here, we found 128 pTyr-containing proteins with 143 pTyr sites (Table S3). Among them, 14 proteins contained more than one pTyr site. LOC_Os06g15820, containing a NHL repeat, was found to have three pTyr sites (Table S3). In addition, five rice MAPKs, OsMPK1, OsMPK3, OsMPK4, OsMPK11 and OsMPK14, were phosphorylated on the conserved Tyr residues in the 'TDY' or 'TEY' motif (Figure S3 and Table S3), which were shown to be phosphorylated by MAPKKs in animals and humans (Fu *et al.*, 2005). Therefore, our findings suggest that Tyr phosphorylation could play similar roles in MAPK signaling cascades during RAM development.

Prediction of cellular localization and functions for RAM proteome and phosphoproteome

For a bioinformatic overview of the subcellular localizations for the proteins identified by our proteome and phosphoproteome, we performed gene ontology (GO) annotation based on cellular component (http://bioinfo.cau.edu.cn/agriGO/analysis.php). The distribution patterns for proteome and phosphoproteome were similar (Figures 1c and S4), with the largest proportion assigned to the cytoplasm, followed by membranes and the nucleus. Notably, 52 and 44 chromosome-associated proteins were found in proteome and phosphoproteome, respectively (Figures 1c and S4 and Table S4). Considering most critical players in meiosis are associated with chromosomes, those proteins would be excellent candidates for investigating molecular mechanisms controlling meiosis at both protein and post-translational modification levels.

Functional categorization enrichment of our proteome and phosphoproteome were performed using MapMan analyses (Usadel *et al.*, 2006) to predict molecular functions. Various molecular functions were enriched ($P < 1.0E-02$) in the RAM proteome, including DNA, amino acid metabolism, protein, cell, glycolysis, TCA, nucleotide metabolism, redox and lipid metabolism (Figure S6a). Conversely, in the RAM phosphoproteome, four categories containing RNA, DNA, cell and signaling were remarkably overrepresented ($P < 1.0E-02$) (Figure S5), revealing the most active processes regulated by phosphorylation in RAM. Further analysis for the detailed information of the overrepresented categories revealed that nine functional processes were enriched ($P < 1.0E-02$) in both phosphoproteome and proteome (Figures 1d and S6b). Although 'RNA regulation of transcription' was enriched in both datasets, its significance was 10^7 times higher in the phosphoproteome than that in the proteome, indicating that protein phosphorylation extensively participates in transcriptional regulation in RAM. In addition, four other functional processes were solely overrepresented in the phosphoproteomic dataset, including DNA repair, protein post-translational modification, calcium signaling and MAPK signaling (Figure 1d). As DNA repair is essential for meiotic recombination and important for gamete formation, we thought that the identification of phosphorylated proteins and their phosphorylation sites in this process can accelerate the studies in molecular mechanisms at the post-translational regulation level. However, the other three processes are mainly involved in signaling transduction pathways, which are largely dependent on post-translational regulation (especially protein phosphorylation) rather than translational regulation. Given that active cell-to-cell communication tightly controls the coordinated development of rice anther, those phosphoproteins and phosphorylation sites can facilitate studies to reveal functional and regulatory mechanisms in signal transduction pathways at the protein modification level.

Additionally, four categories involved in metabolic processes were enriched only in the RAM proteome (Figure S6b). Forty-six proteins involved in 'amino acid metabolism synthesis' were putatively associated with

reactive oxygen species (ROS) metabolism (Table S5). Even though this category was not overrepresented in the phosphoproteome, six ROS associated proteins were found to be phosphorylated (Table S5). Since ROS has emerged as a potentially important signals regulating anther development in rice (Hu *et al.*, 2011), our findings indicated that the metabolism of ROS in RAM is likely regulated by protein phosphorylation. Moreover, two categories including 'protein aa activation' and 'protein folding' were also enriched in the proteome, suggesting that other post-translational events contribute to anther development.

Meiotic and putatively meiotic proteins

During meiosis, homologous chromosome pairing, synapsis, recombination and segregation are the key events, whose normal progress ensures the formation of functional gametes. Studies have revealed that protein phosphorylation is critically involved in meiosis. For example, phosphorylation of the yeast double-stranded break (DSB)-promoting factor Mer2 by Dbf4-dependent Cdc7 kinase (DDK) regulates meiotic recombination (Murakami and Keeney, 2014b) and the prolonged phosphorylation of the *C. elegans* nuclear envelope protein SUN-1 was associated with defects in synapsis and meiotic recombination (Woglar *et al.*, 2013). However, although many genes have been revealed to be functionally involved in meiosis in plants (Luo *et al.*, 2014), very few phosphorylation sites have been revealed to regulate meiotic protein function. Here, our phosphoproteome identified 23 pSer and two pThr sites from 16 non-redundant phosphopeptides, covering 10 known meiotic proteins (Table 1); these proteins are important for chromosome condensation (MEL1), homolog pairing and synapsis (PAIR2 and PAIR3), DNA DSB repair (RAD51C and OsMER11), crossover formation (RPA2C and RFC5) and chromosome segregation (RAD21-4 and BRK1) (Table 1). Surprisingly, we found that OsMEL1 has six phosphorylated sites (five pSer and one pThr) in the same peptide, indicating that these sites were simultaneously phosphorylated during rice meiosis. MEL1 is a germ cell-specific ARGONAUTE (AGO) protein and regulates meiotic chromosome condensation through affecting histone H3 lysine-9 dimethylation in pericentromere regions (Nonomura *et al.*, 2007). A recent high-throughput sequencing data revealed that a large number of phasiRNAs generated from intergenic non-coding RNAs were associated with the activity of MEL1 (Komiya *et al.*, 2014), showing small RNAs pathways involved in rice meiosis. However, protein phosphorylation regulating small RNAs pathways has not been reported yet in plant meiosis. Therefore, the six phosphorylated amino acid residues presented in MEL1 not only suggested its molecular function is potentially regulated by complex phosphorylation modification at multiple sites, but also provide an entry for investigating the underlined biochemical mechanism of protein phosphorylation controlling small RNAs pathways.

To find more phosphorylation sites for potential meiosis-related proteins in rice, we searched for rice homologs of known meiotic genes in Arabidopsis and yeast. First, we found that 17 rice homologs of 14 Arabidopsis meiosis proteins were detected in our proteome and/or phosphoproteome. Among them, three were rice SKP1-like homologs, and two were RPA1 homologs (Table 1); further, seven rice homologs, including RCC2, SCC3, RAD21, DNA ligase 1, RFC1, PS1 and RBR1, were phosphorylated. Totally, 26 phosphorylated sites for the above proteins were identified with 23 pSer and 3 pThr sites (Table 1). If the rice proteins have similar functions to those of their Arabidopsis homologs, the phosphorylation might affect meiotic processes such as chromosome condensation, synapsis, meiotic recombination and spindle orientation (Chelysheva *et al.*, 2005; Ganguly *et al.*, 2005; Lam *et al.*, 2005; Wang *et al.*, 2012a). The largest number of phosphorylation sites in one protein is nine, which were distributed in five different phosphopeptides of PS1, indicating that spindle orientation could be strictly regulated by phosphorylation. Secondly, we identified seven rice homologs of yeast meiotic proteins among our proteome and/or phosphoproteome, (Table 1). Among them, five were phosphorylated, including SMG7, RECA, Kinesin (LOC_Os01g14090), RAD23 and XRCC3, with seven phosphorylated sites (six on Ser and one on Thr; Table 1). These data further extend protein function potentially regulated by phosphorylation in meiosis.

Recently, a proteomic analysis for early rice male meiocytes was published, in which 1316 expressed proteins were identified (Collado-Romero *et al.*, 2014). Comparative analysis with our phosphoproteomic data revealed that 414 meiocytes expressed proteins were phosphorylated (Figure 2a and Table S6). MapMan analysis showed that they were mainly categorized in DNA and protein synthesis, RNA processing and regulation of transcription, protein degradation, targeting and post-translational modification, and signaling (Figure 2b and Table S6). Further analysis found that, except the meiosis-related proteins listed in the Table 1, additional 15 phosphorylated proteins were annotated as chromatin-associated ones (Table S4). Interestingly, five phosphorylated proteins, LOC_Os02g01740 (a putative helicase), LOC_Os03g06220 (a DEAD-box ATP-dependent RNA helicase), LOC_Os02g36710 (a SET-domain-containing protein), LOC_Os03g52310 (a transposon protein) and LOC_Os04g18090 (Histone H1), were exclusively identified in early prophase I stage during meiosis (Collado-Romero *et al.*, 2014). Secondly, 117 phosphorylated proteins were classified into protein synthesis group, including ribosomal protein, initiation and elongation factor sub-groups, in which 52 were identified in rice early meiosis (Table S2 and S6). These results implied that phosphorylation

Table 1 Meiosis-related proteins identified in the proteome and phosphoproteome

MSU ID	Gene name	Proteome/ phosphoproteome	Phosphopeptide	Number of phosphorylation sites	Meiotic processes	Reference
Rice meiosis proteins						
LOC_Os01g67250	*OsRAD21-1*	+/+	QVNNVEGVTEQLT*DNEK	8	Sister chromatid cohesion	Zhang *et al.* (2004)
			GTTES*DGS*NKVGSLTSR			
			KAS*PSS*NDETAK			
			VHES*PVLS*PQRK			
			SAVHPTDMEVDDNQNKDES*AEGYNMEDGPSSHNK			
LOC_Os03g58600	*MEL1*	+/+	YYVEGES*S*DGGS*T*PGS*S*GQAVAR	6	Chromosome condensation	Nonomura *et al.* (2007)
LOC_Os05g50410	*RAD21-4*	−/+	RLS*DVGPTPDLLEEIEPTQTPYEK	2	Chromosome segregation	Zhang *et al.* (2006)
			DYDAFHSDHSVTPGS*PGLSR			
LOC_Os07g32480	*BRK1*	−/+	TS*GFSCVEMQEQR	1		Wang *et al.* (2012a)
LOC_Os04g54340	*OsMRE11*	−/+	SVTAQSNLNS*FS*DDEDTREMLLGAR	2	Processing of meiotic double-strand break	Ji *et al.* (2013)
LOC_Os09g32930	*PAIR2*	+/+	SLAGAGGTQCS*QDK	2	Synaptonemal complex	Nonomura *et al.* (2006)
			S*LAGAGGTQCSQDK			
LOC_Os10g26560	*PAIR3*	−/+	MDNASLQVS*PEAANHGGSAK	1		Wang *et al.* (2011)
LOC_Os01g39630	*Rad51C*	−/+	ANLLAAGYSSLAALSAAS*PPR	1	DNA double-strand break repair	Tang *et al.* (2014)
LOC_Os06g47830	*RPA2C*	−/+	SGAPAPEYSAAGTGAAAAPS*PSKPR	1	Crossover formation	Li *et al.* (2013)
LOC_Os02g53500	*RFC5*	−/+	SLS*FGAK	1		Furukawa *et al.* (2003)
Putative homolog of Arabidopsis meiosis proteins						
LOC_Os04g56720	*RCC2*	+/+	TKDVS*ES*EEDDDEEDDS*DDDENGEIKGGK	4	Chromosome condensation	
			SAS*GEPEKVPAAAEGEEKADGAK			
LOC_Os02g04040	*SMC3*	+/−				
LOC_Os05g09620	*SCC3*	+/+	NLFDGHKS*S*DEES*VSDS* DQQGHGEDNDDDDADQPLINTFR	6		
			SFS*FEPGRLVSLIDNDGK			
			TSS*GFLGGGTSTSTGSLR			
LOC_Os12g44390	*SMC1*	+/−				
LOC_Os08g16610	*RAD21*	−/+	DDNSPFKTPGAGGT*PKSR	1	Processing of meiotic double-strand break	
LOC_Os02g29464	*RAD50*	+/−				
LOC_Os10g34750	*DNA ligase 1*	+/+	ALASESQDIKKPSS*PKK	2	Others	
			TQPQPAAEADGGAVAKPEEKPHS*PVKPK			
LOC_Os05g15520	*RPA1-like*	+/−			Crossover formation	
LOC_Os06g01370	*RPA1-like*	+/−				
LOC_Os05g51060	*PTD*	+/−				

(continued)

Table 1. (continued)

MSU ID	Gene name	Proteome/ phosphoproteome	Phosphopeptide	Number of phosphorylation sites	Meiotic processes	Reference
LOC_Os11g36390	*RFC1*	+/+	TIGLDDDGEEDKMDEDAKT*PSK VAAILEPVGESLPEENGVAS*SEGDEEDSS* DAENNDELVPGDTKPK	3		
LOC_Os07g22680	*SKP1-like*	+/−			Chromosome segregation	
LOC_Os07g43250	*SKP1-like*	+/−				
LOC_Os11g26910	*SKP1-like*	+/−				
LOC_Os09g36830	*ASK2*	+/−				
LOC_Os11g07050	*PS1*	−/+	DSILTDEETSVLS*PEKYDTISPVR EFVHYVAPLNLDYETFS*DNENCVLSVAK HTS*S*LDSTQVNPR IFVAPEDS*ES*EGT*IS*ENLFEISNMK LPFQTLLSNS*PLR	9	Spindle orientation	
LOC_Os11g32900	*RBR1*	−/+	GALLASPS*FGS*K RNTNSQIPGS*PK RVFETLAS*PTK RWPSAGPDGNCYPQS*PK YHPLQATFASPTVSNPVSGNEK	5	DNA synapsis	
LOC_Os08g44860	*MPA1*	+/−				
Putative homolog of Yeast meiosis proteins						
LOC_Os08g21350	*SMG7*	−/+	STS*DIFESLK	1	Chromosome condensation	
LOC_Os01g67510	*RECA*	−/+	SSGT*DS*GEENLSKK	2	DNA strand invasion	
LOC_Os01g14090	*kinesin*	−/+	RLS*LENTGIGK	1	Chromosome segregation	
LOC_Os06g10480	*kinesin*	+/−				
LOC_Os06g15360	*RAD23*	−/+	LVNES*PDS*GAAGGNILGQLAAAMPQALTVTPEER	2	DNA repair	
LOC_Os06g05190	*XRCC3*	−/+	ELVES*DEDQK	1	DNA strand invasion	
LOC_Os03g33580	*BUB3.1*	+/−			Mitotic checkpoint protein	

+/+ indicates that the protein was identified in both proteome and phosphoproteome, +/− indicates that the protein was only identified in proteome, −/+ indicates that the protein was only identified in phosphoproteome,* indicates phosphorylated site.

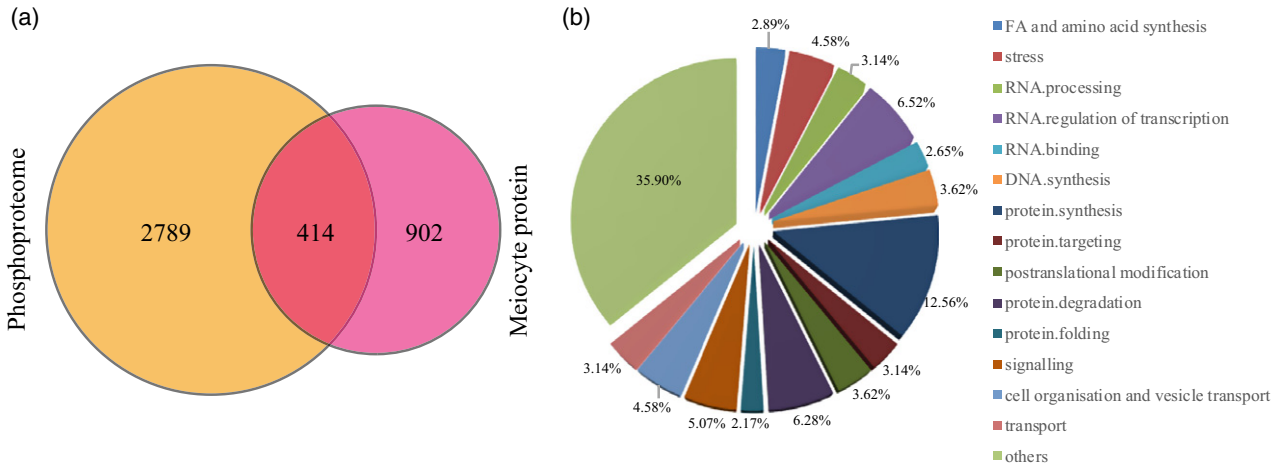

Figure 2. Characteristics of proteins identified in phosphoproteome associated to putative functions involved in meiosis.
(a) Overlap of identified phosphoproteins with reported preliminary meiocyte proteins. In total, 414 meiocyte proteins were phosphorylated.
(b) Distribution of the 414 meiosis-related phosphoproteins across different biological functions (secondary MapMan bins). Only the bins with $P < 1.0E\text{-}02$ of identified proteins are included.

mediated protein synthesis pathways are highly active in early meiosis.

DNA synthesis and RNA splicing pathways

DNA synthesis is an important process both during the mitotic cell cycle and before meiosis and produces two identical replicates from one original DNA molecule. In addition, DNA synthesis is required for meiotic recombination. Interestingly, most of the proteins related to DNA synthesis were identified in our dataset, including all subunits of the mini-chromosome maintenance protein (MCM2-7) complex, which plays a critical role in DNA synthesis initiation (Figure 3a). MCM2, MCM4 and MCM7 were found to be phosphorylated, consistent with the previous report that cyclin-dependent kinases (CDK)-mediated phosphorylation of MCMs affects its loading on chromatin and helicase activation in Xenopus eggs and HeLa cells (Moritani and Ishimi, 2013; Wei *et al.*, 2013; Gao *et al.*, 2014). It was shown that the yeast Dbf4-dependent Cdc7 kinase (DDK) phosphorylates the DSB-promoting factor Mer2 at the replication fork, linking meiotic DNA synthesis with an early prerequisite for DSB formation during meiotic recombination (Murakami and Keeney, 2014a). RPA2 is a member of the replication protein A (RPA) family and was found to be phosphorylated in our dataset; In human U2-OS cells, RPA can be phosphorylated by Cdk2 and checkpoint kinase ATR (ATM and Rad3 related) during replication fork stalling, and its phosphorylation can stabilize the replisome (Murphy *et al.*, 2014).

In addition, we detected phosphorylation of RFC1 and RFC2 in RFC complex and proliferating cell nuclear antigen (PCNA), which are involved in primer interaction and DNA polymerase (Pol) δ recruitment in lagging strand synthesis, respectively (Figure 3a). Moreover, many other effectors involving in lagging strand synthesis were found phosphorylated, such as subunits of Pol α and δ, and DNA ligase 1 (Lig1) which is involved in Okazaki fragments ligation (Figure 3a). All of these results suggested that protein phosphorylation by regulatory kinases in replisomes may be a general mechanism to ensure spatial and temporal coordination of DNA replication with other chromosomal processes. Considering the recent discoveries that lagging strand DNA synthesis plays essential roles in interference-sensitive COs formation by RFC1 mutation analysis (Wang *et al.*, 2012b), we propose that phosphorylation regulates lagging strand synthesis, thus playing important roles in meiotic recombination and possibly other processes. We believe that the discovered proteins and phosphorylation sites could provide valuable information for further investigation of the molecular mechanism of various meiotic processes at genetic and biochemical levels.

RNA splicing removes introns from a transcribed pre-mRNA and alternative splicing serves as a major source for genetic diversity in eukaryotes. It was estimated that 79% of Arabidopsis genes have at least one intron (Schwartz *et al.*, 2008); further analysis revealed that nearly 25% of multi-exon genes experience alternative RNA splicing during flowering (Wang *et al.*, 2014). In mouse, a RNA-seq analysis found more than 13 000 of novel alternative spliced RNAs with some highly expressed in meiocytes (Margolin *et al.*, 2014). Thus RNA splicing might be a critical regulatory mechanism during plant meiosis. In our proteomic and phosphoproteomic datasets, the RNA splicing pathway was greatly overrepresented by KEGG pathways predication. The Arabidopsis spliceosome consists of 107 proteins, 87 rice homologs of Arabidopsis spliceosome proteins were detected in our study and 52 of them were phosphorylated (Figure 3b and Table S7). It was revealed

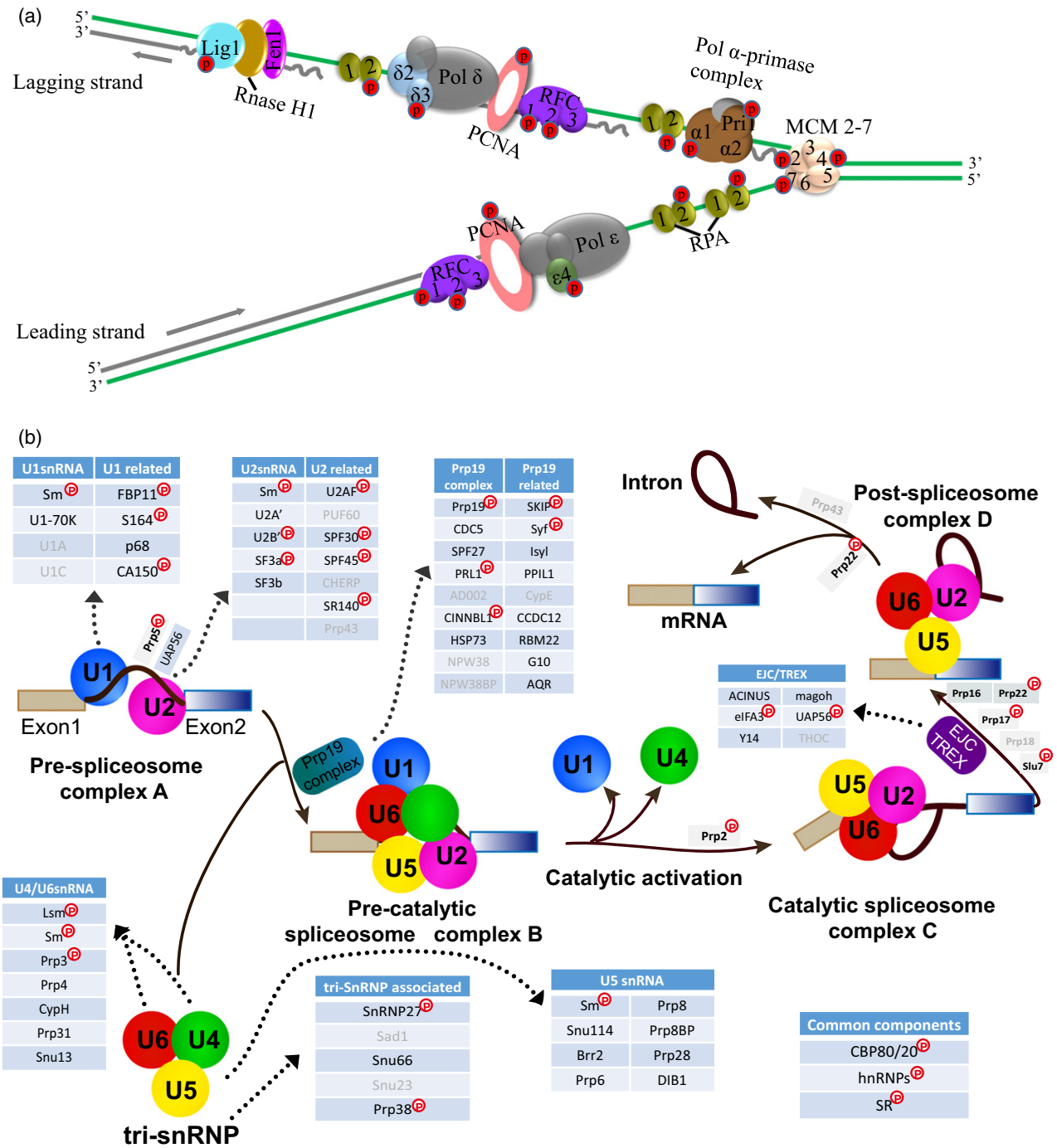

Figure 3. Predicated biological pathways by KEGG of RAM proteome and phosphoproteome.
(a) DNA replication related proteins were phosphorylated. P in a red circle indicates phosphorylated proteins.
(b) Phosphorylation regulates RNA splicing pathway in RAM. Numerous proteins involved in RNA splicing pathway were phosphorylated. Protein names in gray indicate the proteins not observed in our proteomic or phosphoproteomic dataset, P in red circle indicates phosphorylated proteins. The single arrow represents the process of RNA splicing and dotted arrow indicates the element composition of each complex.

that RNA-substrate specificity and interaction, and subcellular localization of Serine/Arginine Rich (SR) proteins are mediated by the phosphorylation of the RS domain in HeLa cells (Misteli *et al.*, 1998). In our results, 13 rice homologs of Arabidopsis SR proteins were phosphorylated, including two of SR1, four of SRZ33, two of SRZ21, one of each of SR45.1, SCI1, SC35-like splicing factor and AT4G36980. Previously reported that phosphorylation of

the N-terminal half of the RS domain catalyzed by the serine kinase SRPK1, regulates the activity of SR1 in constitutive and alternative splicing and modulates the cellular distribution of SR1 (Cazalla *et al.*, 2002; Ngo *et al.*, 2005).

The two rice homologs of SR1, LOC_Os07g47630.1 and LOC_Os03g22380.1, were phosphorylated on serine 88 and serine 123, respectively, in the RNA-recognition motif but not in RS domain; these sites are conserved between rice and Arabidopsis, suggesting functional importance. Moreover, the rice homolog of SRPK1, OsSRPK4 was also found to be phosphorylated on serine 390 in the kinase domain, which implicating that OsSRPK4 may regulate the activity of OsRS1 through phosphorylation in RAM. Our results also revealed that the rice orthologue of SR45.1, LOC_Os01g26940.1, was in a hyperphosphorylated state, containing 17 pSer sites (S5, S86, S100, S193, S228, S284, S296, S301, S307, S323, S320, S355, S392, S433, S481, S583, S603) and one pThr site (T282). These phosphorylation sites are distributed through the RS1, RRM and RS2 domains, supporting a previous model in HeLa cells where RNA to substrate specificity and interaction, and subcellular localization of SR proteins is mediated by the phosphorylation of the RS domain, while the phosphorylation of RRM is required for their recruitment to the sites of transcription (Misteli *et al.*, 1998).

Secretory proteins

Active communications between meiocytes and tapetum mediated by secretory proteins are thought to be important for gametophyte development (Tan *et al.*, 2012). Unfortunately, very few factors were reported to participate in this process, and no phosphorylation regulation was reported in anther development. 507 putative secretory proteins, including 30 previously identified in callus (Jung *et al.*, 2008), were predicted by signalP and targetP in the rice proteome (Table S8). Among 24 fasciclin-like proteins with putative functions in cell–cell communications (Faik *et al.*, 2006), nine proteins including MTR1 (Tan *et al.*, 2012) were identified here, suggesting that multiple fasciclin-like proteins are involved in meiocytes and tapetum communications and coordinately regulate anther development in rice. In addition, 163 putative secretory proteins were phosphorylated (Table S8). Among them, two were cysteine-rich secretory proteins (LOC_Os04g44600 and LOC_Os04g58120), the homolog of Arabidopsis EPIDERMAL PATTERNING FACTOR-LIKE (EPFL) 1 and EPF2, with phosphorylation at six and five sites, respectively. EPFL1 and EPF2 function as ligands of the receptor-like protein kinases ER, ERL1 and ERL2 (Shimada *et al.*, 2011); the phosphorylation of the rice homologs might affect their secretion and affect the interaction with its receptors. Moreover, two phosphorylation sites was identified in the N-terminal of OsTDL1B (LOC_Os10g14020), the homolog of Arabidopsis TPD1 (Table S8); however, these phosphorylation sites were not conserved between OsTDL1A and TPD1, indicating possible differential regulation of OsTDL1B in rice (Zhao *et al.*, 2008).

Protein kinases and phosphatases

Protein kinases and phosphatases mediate reversible protein phosphorylation, playing important roles in plant development (Ganguly *et al.*, 2012; Yang *et al.*, 2015). Kinases themselves are usually regulated by phosphorylation either through autophosphorylation or by other kinases (Bogre *et al.*, 2003; Park *et al.*, 2011; Oh *et al.*, 2012). Here, we found that protein kinases were greatly overrepresented in our phosphoproteome (7.0% (phosphorylated kinases to identified total proteins) compared with 2.2% for the whole predicted proteome (predicated kinases to total proteins in rice protein database)), indicating that phosphorylation broadly exists on the kinases involved in RAM development. In total, 224 kinases with 539 phosphorylation sites were identified in our phosphoproteomic dataset, occupying 15% of 1467 total annotated kinases in rice protein database (Table S9). To determine the kinase families governing the phosphorylation regulatory networks, we performed enrichment analysis for different kinase families. Counting the number of kinases in each family to total number of identified kinases, receptor-like kinases (RLKs) family represented the largest fraction (74, 33%), followed by CAMK (19, 8.5%) and MAPK (12, 5.3%) (Figure 4a and Table S9). In addition, phosphorylated proteins in GSK, URK, CLK, AGC, CK1 and CAMK families showed higher percentages than in other families, implying that these kinase families might be preferentially involved in phosphorylation regulation pathways in RAM. Moreover, 55 phosphorylated phosphatases with 121 phosphorylation sites were detected (Figure 4b and Table S10). Among them, PP2C phosphatases represented the largest fraction (18, 32.7%), followed by PP2A (8, 14.5%), DSP (6, 10.9%) and other phosphatases.

Receptor-like kinases (RLKs) are key components in plant cell signaling, controlling a wide range of plant developmental processes. Nine RLKs, including EMS1, SERK1, SERK2, BAM1, BAM2, ER, ERL1, ERL2 and RPK2, have been revealed to play crucial roles in Arabidopsis anther development (Ma, 2005; Chang *et al.*, 2011). Seventy-four RLKs, belonging to 12 subfamilies, were identified in rice anther phosphoproteome, in which 161 phosphorylation sites were detected (Table S9). Phosphorylated proteins in LRR and RLCK subfamilies exhibited much higher percentages (48 and 25%, respectively) than other subfamilies. Interestingly, among the 36 phosphorylated LRR-RLKs, 15 of them belong to LRR-III and five were classified into LRR-V subfamilies (Table S9). Further analysis revealed that the five members of the LRR-V subfamily are rice homologs of the Arabidopsis STRUBBELIG-RECEPTOR FAMILY (SRF) (SRF3/6/7) members, which are involved in male reproductive

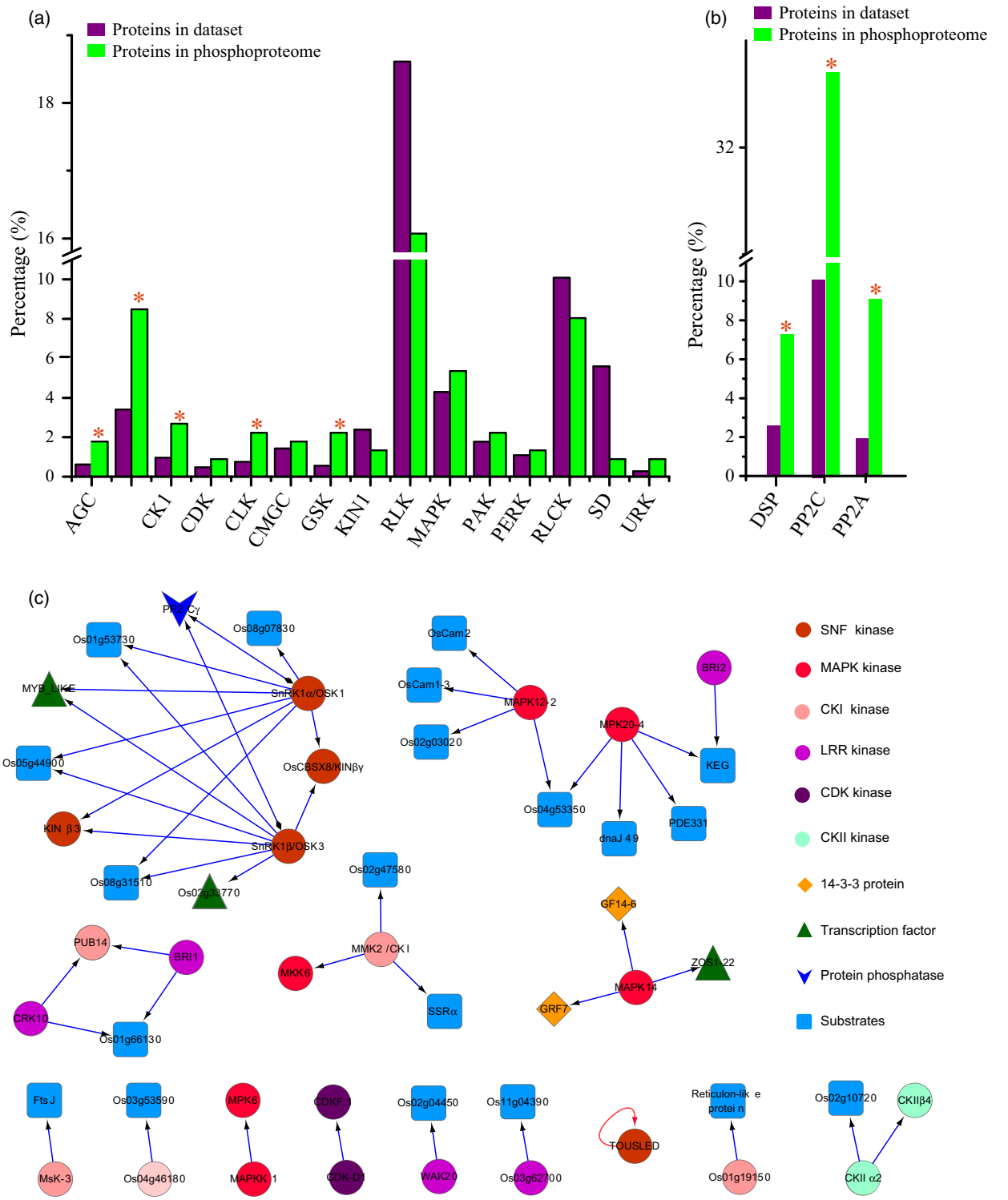

Figure 4. Overview of the kinases and phosphatases identified in phosphoproteome.
(a) The percentage of kinases described in rice protein database and the identified kinases in phosphoproteome. Significantly enriched kinase families in phosphoproteome were identified with red stars (P < 1.0E-02).
(b) The percentage of phosphatases described in rice protein database and the identified phosphatases in phosphoproteome. Compared with their percentage in dataset, DSP, PP2C and PP2A phosphatases showed significantly enriched in phosphoproteome (P < 1.0E-02).
(c) The kinase-substrate network extracted from phosphoproteome. The results are color-coded to indicate the kinase and its substrate where the substrates of SNF1 were found overrepresented. Triangle and arrow represents the phosphorylation regulation. Diamond arrow indicates dephosphorylation regulation. Red circle amd arrow indicates autophosphorylation of kinase.

development (Eyuboglu *et al.*, 2007), suggesting that rice homologs might be also required for anther development and regulated by phosphorylation. Furthermore, as a major regulator of cell cycle, CDKs are functionally regulated by phosphorylation in the T-loop domain, and this modification in AtCDKA-1 was essential for meiosis (Dissmeyer *et al.*, 2007; Guo *et al.*, 2007). Interestingly, four CDKs, including AtCDKA-1 homolog (OsCDKA-1), were found phosphorylated at the conserved Thr residue in the T-loop (Figure S7 and Table S9), providing important evidence for regulation of CDKs function by phosphorylation.

On the other hand, to maintain the dynamic regulation of phosphorylation, phosphatases remove phosphorylation during signal transduction, playing equally important roles to those of kinases. In Arabidopsis, one phosphatase, phosphoserine phosphatase (PSP1) was discovered to participate in male gametophyte development (Cascales-Minana *et al.*, 2013); here we identified the rice homolog of PSP1 (LOC_Os11g41160) in our proteomic dataset, suggesting that PSP1 might have similar function in rice anther development. However, few studies revealed about regulation of phosphatases by phosphorylation during plant anther development. Among 55 identified phosphatases, 11 of them have more than three phosphorylation sites (Table S10), providing direct evidence for phosphorylation as a mechanism regulating the activity of phosphatases. Interestingly, the rice genome encodes five SIT4 phosphatases, whose homologs in yeast were required for the mRNA transcription of G1 cyclin and bud formation (Luke *et al.*, 1996); three of these were phosphorylated at nine sites (Figure S8). Sequence alignment of these three phosphatases showed that they have 70% sequence identity. Moreover, eight phosphorylation sites were highly conserved (Figure S8), indicating that they might share the same regulation mechanism of their activity during anther development.

To further understand the regulatory network mediated by protein phosphorylation, we used the kinase-substrate relationships proposed by the rice Kinase-Protein Interaction Map (Ding *et al.*, 2009). Forty-six such proposed kinase-substrate pairs were found in our phosphoproteome and the interaction network was illustrated with CYTOSCAPE 2.8 software (Figure 4c and Table S11). Substrates of two subunits belonging to SNF1-related protein kinase-1 (SnRK1) family, SnRK1α/OSK1 and SnRK1β/OSK3, were the most highly enriched. SnRK1 is a plant protein kinase complex that regulates numerous metabolic and transcriptional pathways in response to energy deprivation and ABA signals (Polge and Thomas, 2007; Baena-Gonzalez and Sheen, 2008) and contains a catalytic domain similar to that of SNF1 (Sucrose non-fermenting-1) in yeast and AMPK (AMP-activated protein kinase) in animals (Emanuelle *et al.*, 2015). In addition, it was discovered that the barley SnRK1α and SnRK1β are expressed in anthers and the expression of antisense *SnRK1* caused abnormal pollen development and male sterility in barley (Zhang *et al.*, 2001). It has been revealed that calcineurin B-like-interacting protein kinase 15 (CIPK15) serves as an upstream kinase of SnRK1 in rice seedling (Lee *et al.*, 2009) and Arabidopsis PP2Cs dephosphorylate and inactivate SnRK1α (Rodrigues *et al.*, 2013). In our results, an upstream phosphatase PP2Cγ and other three subunits of SnRK1 complex, KINI, OsCBSX8/KINβγ and a DUF581 domain-containing protein (LOC_Os08g31510) were identified. Additionally, two transcription factors (one MYB-like transcription factor (LOC_Os02g57200) and one Triple-Helix transcription factor (LOC_Os02g33770) and three proteins with unknown function were proposed as the substrates of SnRK1. Therefore, SnRK1 might play crucial roles in regulating RAM development through phosphorylating downstream substrates. Moreover, 10 substrates of three MAPKs (MAPK12-2, MAPK14 and MAPK20-4) and upstream kinases of MAPK6 and MKK6 were also uncovered in our results (Figure 4c), showing that phosphorylation of MPKs might also be important for regulating kinase activities in RAM development.

Transcription factors

Transcription factors (TFs) play important roles in regulating gene expression; however, whether and how their activities are controlled by protein modification have not been extensively investigated. Here, we found that 230 TFs were phosphorylated in RAM (Figure 5a and Table S12), indicating that protein phosphorylation is widely involved in modification of TFs, potentially regulating their transcriptional activities. Those phosphorylated TFs were classified into distinct families, including C3H (25, represents 10% of total C3H TFs in predicated total rice proteome), bZIP (23, 10%), PHD (22, 9.5%), MYB (21, 9%) and bHLH (9, 4%) (Figure 5a and Table S13). In addition, subfamilies of BES1, HMG, GIF, GeBp, ZF-HD, SBP, C3H and bZIP showed higher ratio of phosphorylation than others (Figure 5a), implying that these subfamilies may have more important roles during RAM development. In the nine phosphorylated bHLH TFs, three (UDT1, TDR and TIP2) have been shown genetically to be involved in anther development and male fertility (Jung *et al.*, 2005; Li *et al.*, 2006; Fu *et al.*, 2014). The identified phosphorylation sites provide valuable information for further investigation of their functions at protein modification level. For the 23 phosphorylated bZIP TFs, 88 phosphorylation sites were detected and 31 of these sites are evolutionarily conserved in monocot, dicot and *Amborella tricopoda* (Table S12). Interestingly, we found that more members in IX (8 ones) and VI (7 ones) subfamilies were phosphorylated (Table S12), suggesting that phosphorylation may directly affect the activities of the members in these groups during anther development. Similarly, among the 24 phosphorylated C3H TFs, all three

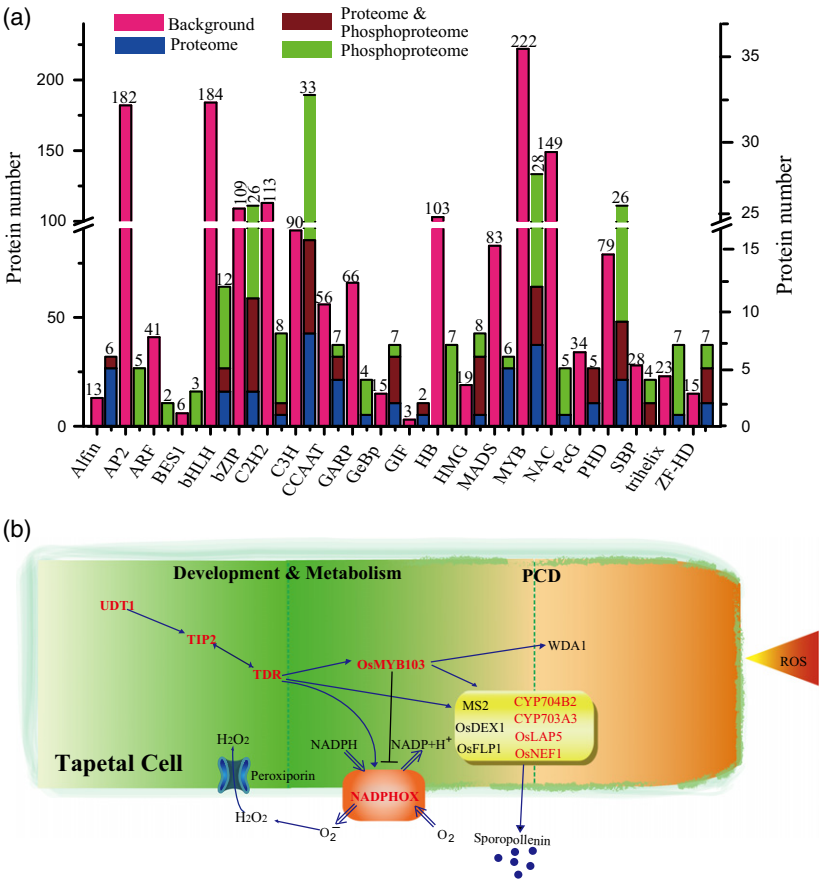

Figure 5. Overview of the transcription factors and UDT1 mediated pathway identified in phosphoproteome and proteome.
(a) The number of transcription factors described in rice protein database and the identified numbers in proteome and phosphoproteome. The results showed that the phosphorylation patterns differ from transcription factor families in RAM.
(b) Phosphorylation affects UDT1 mediated tapetum development pathway. Protein names in red represent phosphorylated proteins identified in RAM and the single arrow represents the gene regulation.

members in the V subfamily (OsC3H22, OsC3H23 and OsC3H53) were phosphorylated (Table S12), implying that members of this subfamily may function as similar regulators with phosphorylation modification controlling rice anther development.

In this study, our proteomic and phosphoproteomic datasets have identified 19 proteins known to be involved in rice anther development; nine of these proteins were phosphorylated at 16 sites (Table S13). Interestingly, the OsMADS3 protein, which is important for stamen identity specification and anther development (Hu *et al.*, 2011), had four detected phosphorylated sites in the same peptide (Table S13), suggesting that multiple modifications could control its activity. However, for TIP2/bHLH142 involved in the tapetum programmed cell death process (Fu *et al.*, 2014), two phosphorylation sites were identified in two distinct peptides (Table S13), raising the possibility that phosphorylation at different sites might have different functional effects.

It has been known that DYT1, AMS, and MYB103 play essential roles in tapetum development and function in Arabidopsis (Sorensen *et al.*, 2003; Zhang *et al.*, 2006, 2007). Interestingly, the rice homologs of these TFs, UDT1, TDR, and OsMYB103, which are also important for rice tapetum development, were found to be phosphorylated at six sites (Table S13), indicating that protein phosphorylation plays an important role in regulating tapetum development in RAM (Figure 5b). In Arabidopsis, tapetum-specific NADPH oxidases (RBOHE) was recently reported to be critical for tapetal programmed cell death (PCD) and its expression was regulated by AMS and MYB103, indicating that the temporal ROS production was important for synchronization between tapetal PCD and pollen development (Xie *et al.*, 2014). In our dataset, the rice NADPH oxidases (OsRBOHE, LOC_Os08g35210) was phosphorylated (Figure 5b). This suggested that UDT1 (Arabidopsis DYT1 homolog) mediated genetic pathway might regulate ROS homeostasis and phosphorylation of OsRBOHE may modulate its NADPH oxidase activity to control ROS levels during tapetum development (Figure 5b).

RNA-directed DNA methylation (RdDM) pathway

In plants, RdDM is a major small RNA-mediated epigenetic pathway, in which two different plant-specific RNA polymerases (Pol IV and Pol V) are recruited to comprise a specialized transcriptional machinery with a number of accessory factors (Matzke *et al.*, 2015). We identified most factors in the RdDM pathway in RAM (Figure 6 and Table S14), indicating that this pathway plays important roles during anther development. Dicer, a key component

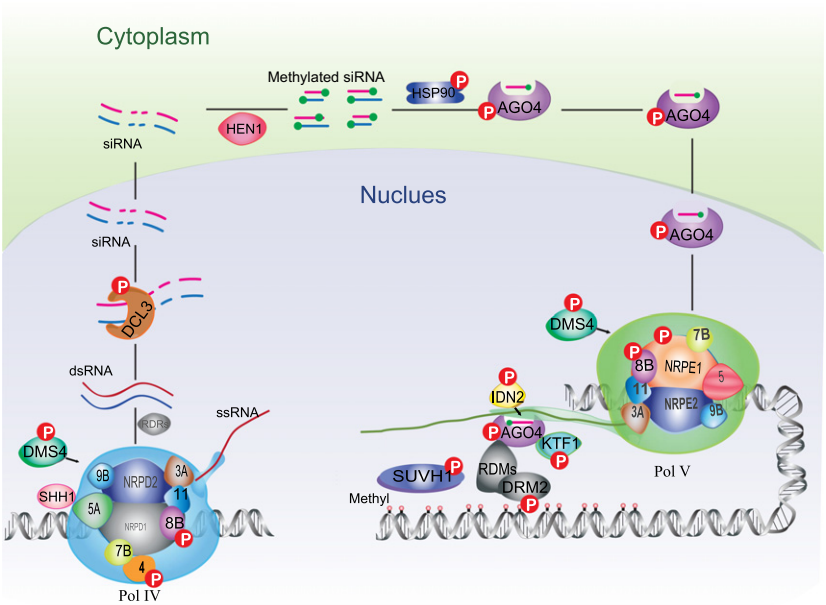

Figure 6. Phosphorylation affects RNA-directed DNA methylation pathway in RAM.
The molecular mechanism for RdDM involves two steps: biogenesis of the 24 nt siRNAs and the conversion of siRNA signals into *de novo* DNA methylation at the target DNA regions. Each step requires a plant-specific DNA-dependent RNA polymerase, abbreviated as Pol IV and Pol V, respectively. P in a red ball represents phosphorylated proteins and detailed protein information is showed in the Table S14.

in small RNA biogenesis, belongs to the RNase III family and cleaves double-stranded RNA (dsRNA) and pre-microRNA (pre-miRNA) into short double-stranded RNA fragments. It was shown that phosphorylation of Dicer in *C. elegans* can suppress its activity in the germline (Drake *et al.*, 2014). Phosphorylation of the human AGO2 at the highly conserved tyrosine Y529 could affect its binding efficiency for small RNAs (Rudel *et al.*, 2011). These results showed that protein phosphorylation is involved in the regulation of RdDM pathway. However, in plants, protein phosphorylation of factors in the RdDM pathway has not been reported yet. Excitingly, our phosphoproteomic data here successfully identified 37 factors that are phosphorylated at 155 sites, including DOMAINS REARRANGED METHYLASE 2 (OsDRM2), Dicer-like 3 (DCL3), Argonaute 4 (AGO4) and NRPD 4 (Figure 6 and Table S14). We identified 13 AGO proteins with nine being phosphorylated (Figure S9 and Table S14), 15 Snf2 proteins (functioning as ATP-dependent chromatin remodeling factors) with 13 being phosphorylated (Table S14). Furthermore, we also detected phosphorylation on three other chromatin remodeling factors, the rice homologs of KOW DOMAIN-CONTAINING TRANSCRIPTION FACTOR 1 (KTF1), INVOLVED IN DE NOVO 2 (IDN2) and DEFFECTIVE IN MERISTEM SILENCING 4 (DMS4), which were shown to stabilize siRNA and scaffold RNA in RdDM pathway (Ausin *et al.*, 2009; Bies-Etheve *et al.*, 2009) (Figure 6). The WG/GW motif of KTF1 was responsible for binding to the AGO4-siRNA complex (Bies-Etheve *et al.*, 2009), and the phosphorylation site Ser-120 in WG/GW motif of rice KTF1 homolog (LOC_Os05g43060) detected in our phosphoproteomic dataset may affect the binding of the AGO4-siRNA complex. In addition, the N-terminal XS domain of IDN2 interacts with dsRNA in RdDM (Ausin *et al.*, 2009). Three phosphorylation sites (Ser 5, Ser 9 and Ser 11) in the N-terminal XS domain of rice IDN2 homolog (LOC_Os01g44230) were detected, which may affect its interaction with dsRNA.

Comparative transcriptional analysis in maize germinal (pre-meiotic) and somatic cells revealed that genes in the RdDM pathway, including *AGO4* and *IDN2,* were preferentially enriched in germinal cell (Kelliher and Walbot, 2014). Thus, we speculated that AGO4/siRNA mediated RdDM pathway might mainly function in rice meiocytes through regulating major meiotic processes. As previously mentioned OsMEL1 (OsAGO4), it is reported that MEL1-phasiRNA complex might regulate meiotic chromosome structure or modification through RNA silencing (Komiya *et al.*, 2014). The phosphorylation status of MEL1 revealed here open a hypothesis that the activity of MEL1 might be controlled by phosphorylation.

CONCLUSION

In flowering plants, anther development near meiosis is a special reproductive stage, in which meiosis generates haploid cells and somatic cells, especially the tapetum, provide nutrients to support meiosis and pollen development. Characterization of the proteome and phosphoproteome of RAM will not only facilitate studies of meiotic processes, such as homolog pairing, meiotic recombination and chromosome segregation, but also enhance researches in protein regulatory networks controlling anther development. Therefore, the data obtained from rice meiotic stage anthers here provide a rich resource for investigating the role of reversible phosphorylation in meiosis and anther development, and improve our understanding of the molecular mechanisms on male fertility.

EXPERIMENTAL PROCEDURES

Materials

Modified trypsin was purchased from Promega (Madison, WI, USA). Oligo R3 reverse phase (RP) material was from PerSeptive Biosystems (Applied Biosystems/Life Technologies, Foster City, CA, USA). Pure water was obtained from a Milli-Q system (Millipore, Bedford, MA, USA). NH_3•H_2O were from Sigma-Aldrich (St. Louis, MO, USA). GELoader tips were from Eppendorf (Hamburg, Germany). 3M Empore C8 disks were from 3M Bioanalytical Technologies (St. Paul, MN, USA). All other reagents and solvents were of the highest commercial quality and were used without further purification.

Plant materials and meiotic stage anther collection

Rice plants (*Oryza sativa* subsp. i*ndica cv. 9311*) were grown in plastic planting bins in a controlled environment chamber (12-h light/12-h dark, 28°C, and 70% relative humidity). Panicle initiation and development was determined by microscopic examination of the appearance of panicle primordial. Florets with 3–5 mm length were collected and stages 5–8 anthers were isolated on a glass slide under a stereoscopic microscope, immediately frozen in liquid nitrogen and stored at −80°C until use.

Total protein extraction and trypsin digestion

Rice anthers were ground to a fine powder in liquid nitrogen, and suspended with 6 M urea and 2 M thiourea 100 mM NH_4HCO_3 (final concentration). The suspension was sonicated for 35 min (2 sec sonication with 5 sec intervals) and the supernatant was collected by centrifugation at 20 000 ***g*** for 20 min. The protein concentration was determined using the Bradford assay. Subsequently, the proteins were submitted to reduction by 5 mM DTT at 37°C for 1 h and alkylation by 15 mM iodoacetamide in dark at room temperature for 1 h. The excess iodoacetamide was quenched by the addition of 5 mM DTT. Finally, the protein digestion was accomplished by Lys-C digestion (1 μg endoproteinase Lys-C for 100 μg protein) for 4 h at room temperature and followed trypsin digestion (1 μg trypsin for 50 μg protein) overnight at 37°C after six volumes dilution with water.

Phosphopeptide enrichment by TiO_2 micro-column

Peptide mixture from 400 μg protein (around 1/5 of total protein) was used for phosphoproteomic analysis. Phosphopeptides were enriched using TiO_2 micro-column as described by Thingholm *et al.* (Thingholm *et al.*, 2006). The digests were vacuum dried and resuspended with TiO_2 loading buffer (1 M glycolic acid in 80% ACN, 1% TFA) and applied onto the TiO_2 micro-column. After washing four times with 20 μl loading buffer and twice with 20 μl washing buffer (80% ACN, 1% TFA), the bound peptides were eluted twice with 20 μl elution buffer 1 (2 M NH_3•H_2O), and with 2 μl elution buffer 2 (1 M NH_3•H_2O in 40% ACN).

Desalting

Protein digests or the eluates from TiO_2 enrichment was acidified with formic acid (FA) and loaded onto a pre-equilibrated homemade Poros R3 micro-column (Gobom *et al.*, 1999). To minimize sample loss, the length of the column was varied depending on the sample amount (approximately 1 μl bed volume of R3 material per 10 μg of sample). After washing the R3 resin twice with 5% FA, the bound peptides were eluted by 15 μl of 30% ACN, followed by 15 μl of 60% ACN.

Nano UHPLC-MS/MS analysis

LC-MS/MS analysis was performed using an LTQ-Orbitrap Elite (ThermoFisher) coupled with an Easy nLC 1000. The LC solvents are 0.1% FA in H_2O (solvent A) and 0.1% FA in 95% ACN (solvent B). The peptide separation was accomplished by three-step elution: 2–35% solvent B in 200 min, 35–90% solvent B in 10 min, 90% solvent B for 5 min, 90–2% solvent B in 2 min, and 2% for 13 min. The peptide ions were detected in the Orbitrap mass spectrometer and up to 15 of the most intense peptide ions (>5000 counts) were selected and fragmented by MS/MS using multistage activation (MSA) in the linear ion trap (Palumbo and Reid, 2008).

Database search and data analysis

Raw data were processed using PROTEOME DISCOVERER software (Version 1.4, Thermo Fisher, Germany) and searched against the TIGR Rice (Oryza sativa Japonica) protein sequence database (FASTA data downloaded from ftp://ftp.plantbiology.msu.edu/pub/data/Eukaryotic_projects/O_Sativa/annotation_dbs/pseudomolecules/version 7.0/all.dir/all.pep; 2010; 66 495 sequences) using an in-house Mascot server (Version 2.3.02, Matrix Science, London, UK). The following parameters were specified in the protein database searches: only tryptic peptides with up to two missed cleavage sites were allowed; 10 ppm mass tolerances for MS and 0.6 Da for MS/MS fragment ions; carbamidomethylcysteine as a fixed modification; and protein *N*-acetylation, oxidized methionine, and phospho_STY (serine, threonine, and tyrosine) permitted as variable modifications (Ye *et al.*, 2010). Peptide identification was achieved by using the following requirements: the expectation value (p) was lower than 0.05 and the peptides were ranked as No. 1 by the Mascot search engine (Matrix Science). Decoy database searches in Mascot revealed a false positive rate lower than 1% at peptide level. Identified peptides were further validated with Target Decoy PSM validator and phosphorylation sites were evaluated with phosphoRS3.0.

The best matched Arabidopsis homologs of identified rice proteins were retrieved through PPDB (http://ppdb.tc.cornell.edu/). Functional classification of identified rice proteins were facilitated by using the best matched Arabidopsis homolog accessions through online MapMan analysis (http://mapman.mpimp-golm.mpg.de/general/ora/ora.shtml). Pathway analysis of identified rice proteins were performed by online software KEGG (http://www.kegg.jp/kegg/pathway.html) using the best matched Arabidopsis homolog accessions. The MS proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with identifier PXD002756 (Vizcaino *et al.*, 2014).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Morphology of rice panicle, another section and workflow of the integrated proteomic and phosphoproteomics.

Figure S2. A correlation analysis between the mRNA expression and protein abundance for 1650 genes; and distribution of identi-

fied phosphorylated serine, threonine, and tyrosine residues in the phosphoproteomic dataset.

Figure S3. Protein sequence alignment of rice MAPKs identified in phosphoproteome.

Figure S4. The pie chart of the subcellular localization of rice anther proteins from the proteomic analysis.

Figure S5. The enriched MapMan bins (bin numbers and bin names) of identified phosphoproteins.

Figure S6. Functional classes assigned to the protein identified in proteome.

Figure S7. Alignments of seven rice cyclin-dependent kinases (CDKs).

Figure S8. Alignments of three rice SIT4 phosphatases.

Figure S9. Phylogenetic classification of rice AGO proteins.

Table S1. The list of identified proteins in the rice meiotic stage anther proteome.

Table S2. The list of identified phosphoproteins in rice meiotic stage anther phosphoproteome.

Table S3. The list of identified proteins with tyrosine phosphosites.

Table S4. The list of putative chromosome-associated proteins, which are identified in our proteome and phosphoproteome.

Table S5. The list of ROS related proteins identified in our proteome and phosphoproteome.

Table S6. The list of 414 identified meiosis-related phosphoproteins.

Table S7. Identified proteins (including phosphoproteins) involved in spliceosome pathway predicted by KEGG.

Table S8. Secretory proteins identified in proteome according to signal P and target P prediction.

Table S9. Overview of phosphorylated RLK.

Table S10. Overview of phosphatase identified in our proteome and phosphoproteome.

Table S11. The identified kinase-substrate interaction identified in phosphoproteome.

Table S12. Overview of transcription factors identified in RMSAs phosphoproteome.

Table S13. Anther development related proteins identified in the proteome and phosphoproteome.

Table S14. Overview of epigenetic related proteins from proteome and phosphoproteome.

REFERENCES

Ausin, I., Mockler, T.C., Chory, J. and Jacobsen, S.E. (2009) IDN1 and IDN2 are required for *de novo* DNA methylation in *Arabidopsis thaliana*. *Nat. Struct. Mol. Biol.* **16**, 1325–1327.

Baena-Gonzalez, E. and Sheen, J. (2008) Convergent energy and stress signaling. *Trends Plant Sci.* **13**, 474–482.

Bai, B., Wu, J., Sheng, W.T., Zhou, B., Zhou, L.J., Zhuang, W., Yao, D.P. and Deng, Q.Y. (2015) Comparative analysis of anther transcriptome profiles of two different rice male sterile lines genotypes under cold stress. *Int. J. Mol. Sci.* **16**, 11398–11416.

Bauernfeind, A.L., Soderblom, E.J., Turner, M.E., Moseley, M.A., Ely, J.J., Hof, P.R., Sherwood, C.C., Wray, G.A. and Babbitt, C.C. (2015) Evolutionary divergence of gene and protein expression in the brains of humans and chimpanzees. *Genome Biol. Evol.* **7**, 2276–2288.

Bies-Etheve, N., Pontier, D., Lahmy, S., Picart, C., Vega, D., Cooke, R. and Lagrange, T. (2009) RNA-directed DNA methylation requires an AGO4-interacting member of the SPT5 elongation factor family. *EMBO Rep.* **10**, 649–654.

Bogre, L., Okresz, L., Henriques, R. and Anthony, R.G. (2003) Growth signalling pathways in Arabidopsis and the AGC protein kinases. *Trends Plant Sci.* **8**, 424–431.

Brar, G.A., Kiburz, B.M., Zhang, Y., Kim, J.E., White, F. and Amon, A. (2006) Rec8 phosphorylation and recombination promote the step-wise loss of cohesins in meiosis. *Nature*, **441**, 532–536.

Cascales-Minana, B., Munoz-Bertomeu, J., Flores-Tornero, M., Anoman, A.D., Pertusa, J., Alaiz, M., Osorio, S., Fernie, A.R., Segura, J. and Ros, R. (2013) The phosphorylated pathway of serine biosynthesis is essential both for male gametophyte and embryo development and for root growth in Arabidopsis. *Plant Cell*, **25**, 2084–2101.

Cazalla, D., Zhu, J., Manche, L., Huber, E., Krainer, A.R. and Caceres, J.F. (2002) Nuclear export and retention signals in the RS domain of SR proteins. *Mol. Cell. Biol.* **22**, 6871–6882.

Chang, F., Wang, Y., Wang, S. and Ma, H. (2011) Molecular control of microsporogenesis in Arabidopsis. *Curr. Opin. Plant Biol.* **14**, 66–73.

Chelysheva, L., Diallo, S., Vezon, D. *et al.* (2005) AtREC8 and AtSCC3 are essential to the monopolar orientation of the kinetochores during meiosis. *J. Cell Sci.* **118**, 4621–4632.

Cheng, Z. (2013) Analyzing meiotic chromosomes in rice. *Methods Mol. Biol.* **990**, 125–134.

Clyne, R.K., Katis, V.L., Jessop, L., Benjamin, K.R., Herskowitz, I., Lichten, M. and Nasmyth, K. (2003) Polo-like kinase Cdc5 promotes chiasmata formation and cosegregation of sister centromeres at meiosis I. *Nat. Cell Biol.* **5**, 480–485.

Collado-Romero, M., Alos, E. and Prieto, P. (2014) Unravelling the proteomic profile of rice meiocytes during early meiosis. *Front Plant Sci.* **5**, 356.

Deveshwar, P., Bovill, W.D., Sharma, R., Able, J.A. and Kapoor, S. (2011) Analysis of anther transcriptomes to identify genes contributing to meiosis and male gametophyte development in rice. *BMC Plant Biol.* **11**, 78.

Ding, X., Richter, T., Chen, M. *et al.* (2009) A rice kinase-protein interaction map. *Plant Physiol.* **149**, 1478–1492.

Dissmeyer, N., Nowack, M.K., Pusch, S., Stals, H., Inze, D., Grini, P.E. and Schnittger, A. (2007) T-loop phosphorylation of Arabidopsis CDKA; 1 is required for its function and can be partially substituted by an aspartate residue. *Plant Cell* **19**, 972–985.

Drake, M., Furuta, T., Suen, K.M., Gonzalez, G., Liu, B., Kalia, A., Ladbury, J.E., Fire, A.Z., Skeath, J.B. and Arur, S. (2014) A requirement for ERK-dependent dicer phosphorylation in coordinating oocyte-to-embryo transition in *C. elegans*. *Dev. Cell* **31**, 614–628.

Emanuelle, S., Hossain, M.I., Moller, I.E. *et al.* (2015) SnRK1 from *Arabidopsis thaliana* is an atypical AMPK. *Plant J.* **82**, 183–192.

Eyuboglu, B., Pfister, K., Haberer, G., Chevalier, D., Fuchs, A., Mayer, K.F. and Schneitz, K. (2007) Molecular characterisation of the *STRUBBELIG-RECEPTOR FAMILY* of genes encoding putative leucine-rich repeat receptor-like kinases in *Arabidopsis thaliana*. *BMC Plant Biol.* **7**, 16.

Faik, A., Abouzouhair, J. and Sarhan, F. (2006) Putative fasciclin-like arabinogalactan-proteins (FLA) in wheat (*Triticum aestivum*) and rice (*Oryza sativa*): identification and bioinformatic analyses. *Mol. Genet. Genomics* **276**, 478–494.

Fu, Z., Schroeder, M.J., Shabanowitz, J., Kaldis, P., Togawa, K., Rustgi, A.K., Hunt, D.F. and Sturgill, T.W. (2005) Activation of a nuclear Cdc2-related kinase within a mitogen-activated protein kinase-like TDY motif by autophosphorylation and cyclin-dependent protein kinase-activating kinase. *Mol. Cell. Biol.* **25**, 6047–6064.

Fu, Z., Yu, J., Cheng, X., Zong, X., Xu, J., Chen, M., Li, Z., Zhang, D. and Liang, W. (2014) The rice basic helix-loop-helix transcription factor TDR INTERACTING PROTEIN2 Is a central switch in early anther development. *Plant Cell* **26**, 1512–1524.

Furukawa, T., Ishibashi, T., Kimura, S., Tanaka, H., Hashimoto, J. and Sakaguchi, K. (2003) Characterization of all the subunits of replication factor C from a higher plant, rice (*Oryza sativa* L.), and their relation to development. *Plant Mol Biol.* **53**, 15–25.

Ganguly, S., Weller, J.L., Ho, A., Chemineau, P., Malpaux, B. and Klein, D.C. (2005) Melatonin synthesis: 14-3-3-dependent activation and inhibition of arylalkylamine N-acetyltransferase mediated by phosphoserine-205. *Proc. Natl Acad. Sci. U. S. A.* **102**, 1222–1227.

Ganguly, A., Sasayama, D. and Cho, H.T. (2012) Regulation of the polarity of protein trafficking by phosphorylation. *Mol. Cells*, **33**, 423–430.

Gao, Y., Yao, J., Poudel, S., Romer, E., Abu-Niaaj, L. and Leffak, M. (2014) Protein phosphatase 2A and Cdc7 kinase regulate the DNA unwinding element-binding protein in replication initiation. *J. Biol. Chem.* **289**, 35987–36000.

Gobom, J., Nordhoff, E., Mirgorodskaya, E., Ekman, R. and Roepstorff, P. (1999) Sample purification and preparation technique based on nano-scale reversed-phase columns for the sensitive analysis of complex peptide mixtures by matrix-assisted laser desorption/ionization mass spectrometry. *J. Mass Spectrom.* **34**, 105–116.

Guo, J., Song, J., Wang, F. and Zhang, X.S. (2007) Genome-wide identification and expression analysis of rice cell cycle genes. *Plant Mol. Biol.* **64**, 349–360.

Hu, L., Liang, W., Yin, C., Cui, X., Zong, J., Wang, X., Hu, J. and Zhang, D. (2011) Rice MADS3 regulates ROS homeostasis during late anther development. *Plant Cell*, **23**, 515–533.

Ji, J., Tang, D., Wang, M., Li, Y., Zhang, L., Wang, K., Li, M. and Cheng, Z. (2013) MRE11 is required for homologous synapsis and DSB processing in rice meiosis. *Chromosoma*, **122**, 363–376.

Jung, K.H., Han, M.J., Lee, Y.S., Kim, Y.W., Hwang, I., Kim, M.J., Kim, Y.K., Nahm, B.H. and An, G. (2005) Rice undeveloped Tapetum1 is a major regulator of early tapetum development. *Plant Cell*, **17**, 2705–2722.

Jung, Y.H., Jeong, S.H., Kim, S.H., Singh, R., Lee, J.E., Cho, Y.S., Agrawal, G.K., Rakwal, R. and Jwa, N.S. (2008) Systematic secretome analyses of rice leaf and seed callus suspension-cultured cells: workflow development and establishment of high-density two-dimensional gel reference maps. *J. Proteome Res.* **7**, 5187–5210.

Kelliher, T. and Walbot, V. (2014) Maize germinal cell initials accommodate hypoxia and precociously express meiotic genes. *Plant J.* **77**, 639–652.

Komiya, R., Ohyanagi, H., Niihama, M., Watanabe, T., Nakano, M., Kurata, N. and Nonomura, K. (2014) Rice germline-specific Argonaute MEL1 protein binds to phasiRNAs generated from more than 700 lincRNAs. *Plant J.* **78**, 385–397.

Lam, W.S., Yang, X. and Makaroff, C.A. (2005) Characterization of *Arabidopsis thaliana* SMC1 and SMC3: evidence that AtSMC3 may function beyond chromosome cohesion. *J. Cell Sci.* **118**, 3037–3048.

Lee, K.W., Chen, P.W., Lu, C.A., Chen, S., Ho, T.H. and Yu, S.M. (2009) Coordinated responses to oxygen and sugar deficiency allow rice seedlings to tolerate flooding. *Sci. Signal.* **2**, ra61.

Leem, S.H. and Ogawa, H. (1992) The MRE4 gene encodes a novel protein kinase homologue required for meiotic recombination in *Saccharomyces cerevisiae*. *Nucleic Acids Res.* **20**, 449–457.

Li, N., Zhang, D.S., Liu, H.S. *et al.* (2006) The rice tapetum degeneration retardation gene is required for tapetum degradation and anther development. *Plant Cell*, **18**, 2999–3014.

Li, X., Chang, Y., Xin, X., Zhu, C., Li, X., Higgins, J.D. and Wu, C. (2013) Replication protein A2c coupled with replication protein A1c regulates crossover formation during meiosis in rice. *Plant Cell*, **25**, 3885–3899.

Luke, M.M., Della Seta, F., Di Como, C.J., Sugimoto, H., Kobayashi, R. and Arndt, K.T. (1996) The SAP, a new family of proteins, associate and function positively with the SIT4 phosphatase. *Mol. Cell. Biol.* **16**, 2744–2755.

Luo, Q., Tang, D., Wang, M., Luo, W., Zhang, L., Qin, B., Shen, Y., Wang, K., Li, Y. and Cheng, Z. (2013) The role of OsMSH5 in crossover formation during rice meiosis. *Mol. Plant*, **6**, 729–742.

Luo, Q., Li, Y., Shen, Y. and Cheng, Z. (2014) Ten years of gene discovery for meiotic event control in rice. *J. Genet. Genomics*, **41**, 125–137.

Ma, H. (2005) Molecular genetic analyses of microsporogenesis and microgametogenesis in flowering plants. *Annu. Rev. Plant Biol.* **56**, 393–434.

Margolin, G., Khil, P., Kim, J., Bellani, M. and Camerini-Otero, R. (2014) Integrated transcriptome analysis of mouse spermatogenesis. *BMC Genom.* **15**, 39.

Matzke, M.A., Kanno, T. and Matzke, A.J. (2015) RNA-directed DNA methylation: the evolution of a complex epigenetic pathway in flowering plants. *Annu. Rev. Plant Biol.* **66**, 243–267.

Mayank, P., Grossman, J., Wuest, S., Boisson-Dernier, A., Roschitzki, B., Nanni, P., Nuhse, T. and Grossniklaus, U. (2012) Characterization of the phosphoproteome of mature Arabidopsis pollen. *Plant J.* **72**, 89–101.

Miao, C., Tang, D., Zhang, H., Wang, M., Li, Y., Tang, S., Yu, H., Gu, M. and Cheng, Z. (2013) Central region component1, a novel synaptonemal complex component, is essential for meiotic recombination initiation in rice. *Plant Cell*, **25**, 2998–3009.

Misteli, T., Caceres, J.F., Clement, J.Q., Krainer, A.R., Wilkinson, M.F. and Spector, D.L. (1998) Serine phosphorylation of SR proteins is required for their recruitment to sites of transcription in vivo. *J. Cell Biol.* **143**, 297–307.

Mithoe, S.C. and Menke, F.L. (2011) Phosphoproteomics perspective on plant signal transduction and tyrosine phosphorylation. *Phytochemistry*, **72**, 997–1006.

Moritani, M. and Ishimi, Y. (2013) Inhibition of DNA binding of MCM2-7 complex by phosphorylation with cyclin-dependent kinases. *J. Biochem.* **154**, 363–372.

Murakami, H. and Keeney, S. (2014a) DDK links replication and recombination in meiosis. *Cell Cycle*, **13**, 3621–3622.

Murakami, H. and Keeney, S. (2014b) Temporospatial coordination of meiotic DNA replication and recombination via DDK recruitment to replisomes. *Cell*, **158**, 861–873.

Murphy, A.K., Fitzgerald, M., Ro, T., Kim, J.H., Rabinowitsch, A.I., Chowdhury, D., Schildkraut, C.L. and Borowiec, J.A. (2014) Phosphorylated RPA recruits PALB2 to stalled DNA replication forks to facilitate fork recovery. *J. Cell Biol.* **206**, 493–507.

Myers, M.V., Manning, H.C., Coffey, R.J. and Liebler, D.C. (2012) Protein expression signatures for inhibition of epidermal growth factor receptor-mediated signaling. *Mol. Cell Proteomics*, **11**, M111 015222.

Ngo, J.C., Chakrabarti, S., Ding, J.H., Velazquez-Dones, A., Nolen, B., Aubol, B.E., Adams, J.A., Fu, X.D. and Ghosh, G. (2005) Interplay between SRPK and Clk/Sty kinases in phosphorylation of the splicing factor ASF/SF2 is regulated by a docking motif in ASF/SF2. *Mol. Cell*, **20**, 77–89.

Nie, L., Wu, G. and Zhang, W. (2006) Correlation of mRNA expression and protein abundance affected by multiple sequence features related to translational efficiency in Desulfovibrio vulgaris: a quantitative analysis. *Genetics*, **174**, 2229–2243.

Nonomura, K., Miyoshi, K., Eiguchi, M., Suzuki, T., Miyao, A., Hirochika, H. and Kurata, N. (2003) The MSP1 gene is necessary to restrict the number of cells entering into male and female sporogenesis and to initiate anther wall formation in rice. *Plant Cell*, **15**, 1728–1739.

Nonomura, K., Nakano, M., Eiguchi, M., Suzuki, T. and Kurata, N. (2006) PAIR2 is essential for homologous chromosome synapsis in rice meiosis I. *J. Cell Sci.* **119**, 217–225.

Nonomura, K., Morohoshi, A., Nakano, M., Eiguchi, M., Miyao, A., Hirochika, H. and Kurata, N. (2007) A germ cell specific gene of the ARGONAUTE family is essential for the progression of premeiotic mitosis and meiosis during sporogenesis in rice. *Plant Cell*, **19**, 2583–2594.

Oh, M.H., Wu, X., Kim, H.S., Harper, J.F., Zielinski, R.E., Clouse, S.D. and Huber, S.C. (2012) CDPKs are dual-specificity protein kinases and tyrosine autophosphorylation attenuates kinase activity. *FEBS Lett.* **586**, 4070–4075.

Olsen, J.V., Vermeulen, M., Santamaria, A. *et al.* (2010) Quantitative phosphoproteomics reveals widespread full phosphorylation site occupancy during mitosis. *Sci. Signal.* **3**, ra3.

Palumbo, A.M. and Reid, G.E. (2008) Evaluation of gas-phase rearrangement and competing fragmentation reactions on protein phosphorylation site assignment using collision induced dissociation-MS/MS and MS3. *Anal. Chem.* **80**, 9735–9747.

Park, H.S., Ryu, H.Y., Kim, B.H., Kim, S.Y., Yoon, I.S. and Nam, K.H. (2011) A subset of OsSERK genes, including OsBAK1, affects normal growth and leaf development of rice. *Mol. Cells*, **32**, 561–569.

Pearlman, S.M., Serber, Z. and Ferrell, J.E. Jr (2011) A mechanism for the evolution of phosphorylation sites. *Cell*, **147**, 934–946.

Polge, C. and Thomas, M. (2007) SNF1/AMPK/SnRK1 kinases, global regulators at the heart of energy control? *Trends Plant Sci.* **12**, 20–28.

Qeli, E. and Ahrens, C.H. (2010) PeptideClassifier for protein inference and targeted quantitative proteomics. *Nat. Biotechnol.* **28**, 647–650.

Rockmill, B. and Roeder, G.S. (1991) A meiosis-specific protein kinase homolog required for chromosome synapsis and recombination. *Genes Dev.* **5**, 2392–2404.

Rodrigues, A., Adamo, M., Crozet, P. *et al.* (2013) ABI1 and PP2CA phosphatases are negative regulators of Snf1-related protein kinase1 signaling in Arabidopsis. *Plant Cell*, **25**, 3871–3884.

Rudel, S., Wang, Y., Lenobel, R., Korner, R., Hsiao, H.H., Urlaub, H., Patel, D. and Meister, G. (2011) Phosphorylation of human Argonaute proteins affects small RNA binding. *Nucleic Acids Res.* **39**, 2330–2343.

Schwartz, S.H., Silva, J., Burstein, D., Pupko, T., Eyras, E. and Ast, G. (2008) Large-scale comparative analysis of splicing signals and their corresponding splicing factors in eukaryotes. *Genome Res.* **18**, 88–103.

Shao, T., Tang, D., Wang, K., Wang, M., Che, L., Qin, B., Yu, H., Li, M., Gu, M. and Cheng, Z. (2011) OsREC8 is essential for chromatid cohesion and metaphase I monopolar orientation in rice meiosis. *Plant Physiol.* **156**, 1386–1396.

Shimada, T., Sugano, S.S. and Hara-Nishimura, I. (2011) Positive and negative peptide signals control stomatal density. *Cell. Mol. Life Sci.* **68**, 2081–2088.

Sorensen, A.M., Krober, S., Unte, U.S., Huijser, P., Dekker, K. and Saedler, H. (2003) The Arabidopsis ABORTED MICROSPORES (AMS) gene encodes a MYC class transcription factor. *Plant J.* **33**, 413–423.

Swindell, W.R., Remmer, H.A., Sarkar, M.K. *et al.* (2015) Proteogenomic analysis of psoriasis reveals discordant and concordant changes in mRNA and protein abundance. *Genome Med.* **7**, 86.

Tan, H., Liang, W., Hu, J. and Zhang, D. (2012) MTR1 encodes a secretory fasciclin glycoprotein required for male reproductive development in rice. *Dev. Cell*, **22**, 1127–1137.

Tang, X., Zhang, Z.Y., Zhang, W.J., Zhao, X.M., Li, X., Zhang, D., Liu, Q.Q. and Tang, W.H. (2010) Global gene profiling of laser-captured pollen mother cells indicates molecular pathways and gene subfamilies involved in rice meiosis. *Plant Physiol.* **154**, 1855–1870.

Tang, D., Miao, C., Li, Y., Wang, H., Liu, X., Yu, H. and Cheng, Z. (2014) OsRAD51C is essential for double-strand break repair in rice meiosis. *Front Plant Sci.* **5**, 167.

Thingholm, T.E., Jorgensen, T.J., Jensen, O.N. and Larsen, M.R. (2006) Highly selective enrichment of phosphorylated peptides using titanium dioxide. *Nat. Protoc.* **1**, 1929–1935.

Usadel, B., Nagel, A., Steinhauser, D. *et al.* (2006) PageMan: an interactive ontology tool to generate, display, and annotate overview graphs for profiling experiments. *BMC Bioinformatics*, **7**, 535.

Vizcaino, J.A., Deutsch, E.W., Wang, R. *et al.* (2014) ProteomeXchange provides globally coordinated proteomics data submission and dissemination. *Nat. Biotechnol.* **32**, 223–226.

Wang, M., Wang, K., Tang, D., Wei, C., Li, M., Shen, Y., Chi, Z., Gu, M. and Cheng, Z. (2010) The central element protein ZEP1 of the synaptonemal complex regulates the number of crossovers during meiosis in rice. *Plant Cell*, **22**, 417–430.

Wang, K., Wang, M., Tang, D., Shen, Y., Qin, B., Li, M. and Cheng, Z. (2011) PAIR3, an axis-associated protein, is essential for the recruitment of recombination elements onto meiotic chromosomes in rice. *Mol. Biol. Cell*, **22**, 12–19.

Wang, M., Tang, D., Luo, Q., Jin, Y., Shen, Y., Wang, K. and Cheng, Z. (2012a) BRK1, a Bub1-related kinase, is essential for generating proper tension between homologous kinetochores at metaphase I of rice meiosis. *Plant Cell*, **24**, 4961–4973.

Wang, Y., Cheng, Z., Huang, J., Shi, Q., Hong, Y., Copenhaver, G.P., Gong, Z. and Ma, H. (2012b) The DNA replication factor RFC1 is required for interference-sensitive meiotic crossovers in *Arabidopsis thaliana*. *PLoS Genet.* **8**, e1003039.

Wang, H., You, C., Chang, F., Wang, Y., Wang, L., Qi, J. and Ma, H. (2014) Alternative splicing during Arabidopsis flower development results in constitutive and stage-regulated isoforms. *Front Genet.* **5**, 25.

Wei, Q., Li, J., Liu, T., Tong, X. and Ye, X. (2013) Phosphorylation of minichromosome maintenance protein 7 (MCM7) by cyclin/cyclin-dependent kinase affects its function in cell cycle regulation. *J. Biol. Chem.* **288**, 19715–19725.

Woglar, A., Daryabeigi, A., Adamo, A., Habacher, C., Machacek, T., La Volpe, A. and Jantsch, V. (2013) Matefin/SUN-1 phosphorylation is part of a surveillance mechanism to coordinate chromosome synapsis and recombination with meiotic progression and chromosome movement. *PLoS Genet.* **9**, e1003335.

Xie, H.T., Wan, Z.Y., Li, S. and Zhang, Y. (2014) Spatiotemporal production of reactive oxygen species by NADPH oxidase is critical for tapetal programmed cell death and pollen development in Arabidopsis. *Plant Cell*, **26**, 2007–2023.

Yang, K.Z., Jiang, M., Wang, M., Xue, S., Zhu, L.L., Wang, H.Z., Zou, J.J., Lee, E.K., Sack, F. and Le, J. (2015) Phosphorylation of serine 186 of bHLH transcription factor SPEECHLESS promotes stomatal development in Arabidopsis. *Mol. Plant*, **8**, 783–795.

Yao, Q., Ge, H., Wu, S., Zhang, N., Chen, W., Xu, C., Gao, J., Thelen, J.J. and Xu, D. (2014) P(3)DB 3.0: from plant phosphorylation sites to protein networks. *Nucleic Acids Res.* **42**, D1206–D1213.

Ye, J., Zhang, X., Young, C., Zhao, X., Hao, Q., Cheng, L. and Jensen, O.N. (2010) Optimized IMAC-IMAC protocol for phosphopeptide recovery from complex biological samples. *J. Proteome Res.* **9**, 3561–3573.

Zhang, Y., Shewry, P.R., Jones, H., Barcelo, P., Lazzeri, P.A. and Halford, N.G. (2001) Expression of antisense SnRK1 protein kinase sequence causes abnormal pollen development and male sterility in transgenic barley. *Plant J.* **28**, 431–441.

Zhang, L.R., Tao, J.Y. and Wang, T. (2004) Molecular characterization of OsRAD21-1, a rice homologue of yeast RAD21 essential for mitotic chromosome cohesion. *J. Exp. Bot.* **55**, 1149–1152.

Zhang, W., Sun, Y., Timofejeva, L., Chen, C., Grossniklaus, U. and Ma, H. (2006) Regulation of Arabidopsis tapetum development and function by DYSFUNCTIONAL TAPETUM1 (DYT1) encoding a putative bHLH transcription factor. *Development*, **133**, 3085–3095.

Zhang, Z.B., Zhu, J., Gao, J.F. *et al.* (2007) Transcription factor AtMYB103 is required for anther development by regulating tapetum development, callose dissolution and exine formation in Arabidopsis. *Plant J.* **52**, 528–538.

Zhao, X., de Palma, J., Oane, R., Gamuyao, R., Luo, M., Chaudhury, A., Herve, P., Xue, Q. and Bennett, J. (2008) OsTDL1A binds to the LRR domain of rice receptor kinase MSP1, and is required to limit sporocyte numbers. *Plant J.* **54**, 375–387.

Zuo, J. and Li, J. (2013) Molecular dissection of complex agronomic traits of rice: a team effort by Chinese scientists in recent years. *Natl Sci. Rev.* **1**, 253–276.