Comprehensive Network Analysis of Anther-Expressed Genes in Rice by the Combination of 33 Laser Microdissection and 143 Spatiotemporal Microarrays

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Abstract

Co-expression networks systematically constructed from large-scale transcriptome data reflect the interactions and functions of genes with similar expression patterns and are a powerful tool for the comprehensive understanding of biological events and mining of novel genes. In Arabidopsis (a model dicot plant), high-resolution co-expression networks have been constructed from very large microarray datasets and these are publicly available as online information resources. However, the available transcriptome data of rice is limited so far, making it difficult for rice researchers to achieve reliable co-expression analysis. In this study, we performed co-expression network analysis by using combined 44 K Agilent microarray datasets of rice, which consisted of 33 laser microdissection (LM)-microarray datasets of anthers, and 143 spatiotemporal transcriptome datasets deposited in RiceXPro. The entire data of the rice co-expression network, which was generated from the 176 microarray datasets by the Pearson correlation coefficient (PCC) method with the mutual rank (MR)-based cut-off, contained 24,258 genes and 60,441 genes pairs. Using these datasets, we constructed high-resolution co-expression subnetworks of two specific biological events in the anther, “meiosis” and “pollen wall synthesis”. The meiosis network contained many known or putative meiotic genes, including genes related to meiosis initiation and recombination. In the pollen wall synthesis network, several candidate genes involved in the sporopollenin biosynthesis pathway were efficiently identified. Hence, these two subnetworks are important demonstrations of the efficiency of co-expression network analysis in rice. Our co-expression analysis included the separated transcriptomes of pollen and tapetum cells in the anther, which are able to provide precise information on transcriptional regulation during male gametophyte development in rice. The co-expression network data presented here is a useful resource for rice researchers to elucidate important and complex biological events.

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Introduction

Recent developments in high-throughput microarray, next-generation sequencing, proteome analysis, and the accumulated functional genomics data across species have enabled us to utilize integrated large-scale data of gene-expression, protein-protein interaction, and phenotype. There is now an increasing need for integrated analysis at a system biology level, to gain an understanding of the complex relationships between gene-product interactions and biological events (e.g. phenotype). An in-silico derived co-expression network is constructed from large-scale gene expression profiles, and is based on the assumption that genes with similar expression patterns are likely to interact with each other at the molecular or physiological level. In some model plants as well as animals, this strategy has been broadly used to predict integrated networks in association with protein-protein interaction data [1], the structural information on metabolic pathways [2–4], and the functions of gene products [5].

To date, a model plant, Arabidopsis thaliana, has been subjected to thousands of microarray experiments and the results have been deposited in publicly-available online databases [6]. By using these very large expression datasets, high-resolution co-expression networks have been constructed and these are available as online information resources in Arabidopsis. Such useful information resources have enabled Arabidopsis researchers to identify novel factors involved in cell wall synthesis [3,4] and the aliphatic...
glucosinolate biosynthesis pathway [5]. On the other hand, in a model crop for the grass family, rice (Oryza sativa), there are few resources for co-expression network analysis, because of inadequate proliferation of available rice microarray datasets derived from various different platforms and a low number of datasets with few examples of publicly available data [7]. Moreover, in rice, there have been fewer case studies to estimate the effectiveness of co-expression networks, compared to those in Arabidopsis. Thus, a reliable large-scale study of co-expression network analysis is required to drive forward rice research at this time.

Male gametophyte development might be a good target for co-expression network analysis in rice, because pollen transcriptome analysis can be easily and precisely analyzed using larger rice flowers than those in Arabidopsis. In flowering plants, pollen development takes place within a male reproductive organ, the anther, and is controlled precisely by four sporophytic cell layers of the anther (tapetum, middle layer, endothecium and epidermis), which surround the gametophytic pollen grains [8]. After differentiation of the male germline, pollen mother cells form tetrads of haploid microspores via meiosis, and the tetrads microspores are connected to each other by a callose wall. Tapetum cells play an important role in degradation of the callose wall, which allows the microspores to be released into the anther locule. The released microspores subsequently mature into pollen grains through cell division and pollen wall formation. During the course of pollen maturation, the tapetum starts to degenerate and provides the various materials for pollen wall formation on the surface of pollen grains. So far, by using situ hybridization [9–15] or microarrays [16,17], many genes expressed in anthers have been identified in Arabidopsis [9,13,17], Brassica napus [10], rice [11,12,14], and Lotus japonicus [15,16]. These studies have revealed the complex patterns of gene expression in both the gametophytic pollen/microspore and sporophytic tapetum cells, which are different but influence each other; consequently, it has been difficult to analyze the precise regulatory interactions of gene expression between pollen and tapetum by examination of the whole anther transcriptome. In this context, transcriptome data from separated pollen and tapetum cells are necessary to compare with the large sets of other microarray data in order to achieve co-expression network analysis of male gametophyte development in plants. Recently, we have performed transcriptome analysis using 44 K microarrays with RNAs extracted independently from pollen and tapetum cells within the rice anther by laser microdissection (LM) technology [18,19].

By using our LM-microarray [18,19] and other publicly available microarray datasets [7], in this study, we have conducted comprehensive co-expression analysis and constructed the co-expression subnetworks responsible for two important biological events during anther development, meiosis and pollen wall synthesis. The meiosis network contained many putative meiotic genes as well as known meiotic genes, which could play meiotic roles such as meiosis initiation and recombination. Furthermore, from the pollen wall synthesis network, we efficiently identified several candidate genes involved in the sporopollenin biosynthesis pathway. Taken together, our co-expression network has the potential to be a powerful resource to dissect various important biological events and/or to isolate novel genetic factors regulating development and differentiation of the male reproductive organ in plants at a system biology level. In addition, this study is an important development as it is one of the few demonstrations of the efficiency of co-expression network analysis in rice.

Results

Dataset content

We have previously conducted 44 K agilent LM-microarray analysis of the microspore/pollen and tapetum cells of japonica rice, Oryza sativa cv. Nipponbare, during anther development from meiosis to tricellular pollen stages [18,19]. In addition to the above study, a LM sample in premeciosis stage was prepared in this study, for more precise gene expression profiling during anther development. The overall LM-microarray data, including three novel microarray datasets, has been deposited at the Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/; accession number GSE 29217). A detailed procedure for the LM-microarray is described in our previous reports [18,19].

Recently, co-expression analysis datasets have become a powerful tool to extract the transcriptional networks of genes involved in various biological events in plants [20]. Therefore, we conducted microarray-based co-expression analysis using our LM-microarray data to gain biological insights into gene expression networks mediating rice anther development. Because popular methods that derive regulatory networks from gene expression data, in general, require large amounts of gene expression data, here we used 143 spatiotemporal expression profiles of 48 various vegetative, and reproductive organs and tissues of rice deposited in RiceXPro [7], in addition to our 33 LM-microarray data of rice anthers [18,19] (Table S1).

A 44K agilent microarray slide contains 29,864 distinct rice genes. Using the 176 microarray, first, we examined the spatiotemporal expression pattern by identifying the highest signal intensity for each gene from different stages and organs (Figure 1, Tables S2, S3, S4, S5, S6, S7, S8, S9, S10, S11, S12, S13, S14, S15, S16, S17, S18, S19, S20, S21, S22, S23, S24, S25). Among the 29,864 genes analyzed, about 10,600 genes were found to be expressed preferentially in at least one of the anther developmental stages or tissues (Figure 1, Tables S7, S8, S9, S10, S11, S12, S13, S14, S15, S16, S17, S18, and S19 for array data of genes preferentially expressed in anther). The remaining approximately 19,300 genes were expressed in the other 44 vegetative or reproductive tissues (Tables S2, S3, S4, S5, S6, S20, S21, S22, S23, S24, S25). Such a large proportion of anther preferentially expressed genes indicates that many genes are involved in anther development and, consequently, analysis of co-expression networks of anther preferentially expressed genes could potentially provide good examples to demonstrate the application of microarray datasets.

Biological significance of expression similarity

We first conducted an expression coherence (EC) analysis using gene ontology (GO) terms to examine the biological significance of expression similarity in the 176 microarray datasets used in this study. EC analysis is a statistical analysis to determine whether gene members belonging to a predefined functional gene set are correlated with each other at the transcriptional level [21]. The EC score is a measure of the expression similarity within a set of predefined functional genes [21]; consequently, a higher EC score is obtained, when most of genes with the same GO term are co-expressed with each other. In our EC analysis, the EC scores of all three GO groups (Biological Process, BP; Molecular Function, MF; and Cellular Component, CC) were higher than that expected by random sampling (Figure 2). Among the three GO groups, CC showed the highest expression similarity, in which approximately 17% of categories exhibited higher EC scores than random sampling at threshold EC score of 0.05, while approximately 12% and 8% did so in BP and MF, respectively.
line in Figure 2). For genes annotated according to the BP group, the “photosynthesis, light harvesting” category showed the highest EC scores (EC = 0.868, 17 genes), whereas the scores of the categories “small ribosomal subunit” (EC = 0.433, 21 genes) and “threonine-type endopeptidase activity” (EC = 0.322, 24 genes) were highest in the CC and MF groups (Table S26). These results confirm that the levels of expression similarity estimated from the microarray datasets in this study are good reflections of functional similarity.

Construction of the meiosis-specific network

By using the Pearson correlation coefficient (PCC) method with the mutual rank (MR)-based cut-off (see Materials and Methods), we identified the final dataset of rice co-expression networks generated from all the 176 microarray data used in this study, which contains 24,259 genes (nodes) and 60,449 gene pairs (edges). From this entire dataset of the rice co-expression network, we are able to construct a subnetwork around gene(s) of interest as guide(s).

To examine whether our co-expression network analysis can identify useful transcriptional networks related to anther development, we first constructed a co-expression subnetwork centered on 9 known meiotic genes (guide genes; red circles in Figure 3A), PAIR1 [22], PAIR2 [23], PAIR3 [24], MEL1 [25], OsRAD21–4 [26], DMC1A and 1B [27], OsMER3/OsRCK [28], and OsSDS [28], whose functions in meiosis have been experimentally demonstrated in rice. The expression profiles of these nine genes after normalized by the VSN algorism (Figure S1A) are well corresponding to the previous results of their expression patterns: indicating that the VSN normalization was a suitable method for the construction of co-expression network. The meiosis-specific network consisted of a large main cluster with the 7 guide genes, and two independent minor clusters of OsMER3 and PAIR1 (Figure 3A), which in total contained 187 genes and 346 gene interactions (Table S27). The main cluster also contained ZEP1 (rice ZYP1 ortholog) and MEL2 (blue circles in Figure 3A), which have most recently been reported as rice meiotic genes [29,30]. Moreover, PAIR2 and ZEP1 [23,29], whose products are both constituents of the synaptonemal complex, were directly connected in the network (red bold lines in Figure 3A). Good association of the 7 meiotic guide genes with each other and with the additional 2 meiotic genes in the main expression cluster indicates that genes involved in meiosis could be strictly co-regulated at the expression level, and thus the meiotic events are potentially good subjects for co-expression analysis. This is also supported by the results of GO analysis (Figures 3B and 3C, Table S28), in which DNA-repair-related GO terms (such as “chromosome organization,” “DNA unwinding involved in replication,” “Mismatch repair,” and
Network Analysis of Anther-Expressed Genes in Rice

A

B

C
Network Analysis of Anther-Expressed Genes in Rice

![Figure 3. The meiosis-specific co-expression network in rice. (A) The co-expression subnetwork constructed using 9 reported rice meiotic genes as guide genes (red circles). The network contains the most-recently reported meiotic genes (blue circles), putative meiotic genes (green circles) and other genes (open circles). A red bold line indicates the direct connection of PAIR2 and ZEP1 in the co-expression network. A link between two nodes indicates direct interaction with PCC >0.64 and MR <10. The subnetwork vicinity is extracted by taking 2 steps out from a guide gene. (B, C) Percentage of genes classified in enriched GO terms of BP (B) or MF (C) for the genes in the meiosis-specific network (red) relative to for 11,456 genes (blue) that had at least one connection at the PCC (>0.64) and MR (<10) thresholds. Enrichment analysis is performed using the hypergeometric distribution (P value <0.05).

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“Mismatched DNA binding”) appear frequently in genes in the meiosis-specific network.

Known or putative meiotic genes in the subnetwork are listed in Table 1. The meiosis-specific network contained cyclin genes and other potential rice orthologs of meiotic genes (Figure 3A). In the network, genes related to meiotic recombination (e.g. DMCI and OsMSH4) are located near a core of the network, and genes functioning in other meiotic events (e.g. MEL1, PAIR3, OsRAD21–4, and OsSDS) surround them with the small independent cluster of PAIR1 (Figure 3A). The independent small cluster of OsMERM (a meiotic recombination-related gene) is due to its higher vegetative expression, which could indicate a bifunctional role in meiosis and other biological events.

Among genes related to “transition from mitosis into meiosis” in Table 1, MEL1 and MEL2 encode ARGONAUTE, and RNA-recognition-motif protein, respectively [24,30], and putative OsAML1 (Os01g0907900) also encodes a RNA-binding protein [31], which is highly similar to MEL2 of Schizosaccharomyces pombe [32]. Together with PWWP- and SET-domain proteins (Table 1), MEL1, MEL2 and OsAML1 might regulate the initiation of meiosis in rice through molecular mechanisms related to RNA metabolism, chromatin remodeling and/or transcriptional regulation. On the other hand, genes related to “meiotic recombination” are well integrated in the network (Figure 3A, Table 1). Co-expression of recombination-related genes in the network is consistent with previous reports that DMCIa and DMCIb co-ordinate with RAD17 [33], and that MSH4 and MSH5 function as a complex [34].

In addition, it can be concluded from the GO analysis (Figure 3B and 3C, Table S28) that various additional meiotic genes could be included in the network as unknown genes (open circles in Figure 3A). This indicates that novel meiotic genes could be mined from the present data, and that novel interactions between known meiotic genes could also be identified from the unknown-gene-mediated connections in the network. Meiosis is an extremely specialized biological event, thus co-expression network analysis has the potential to achieve maximum success in the identification of gene interactions.

Construction of the pollen wall synthesis network

As a second example system, we analyzed the pollen wall synthesis network to systematically identify the genes that modulate the formation of the outer pollen wall, the exine. Previous biochemical analyses have shown that the exine layer consists mainly of sporopollenin, a polymer of phenylpropanoid and lipidic monomers covalently linked by ether and ester linkages [35–37]. When three genes for rice sporopollenin biosynthesis or transport (CYP703A3, CYP704B2, and Osc6) were selected as guide genes [38–40], a major subnetwork covering all guide genes was identified, which consisted of 108 genes and 278 gene interactions (Figure 4A, Table S29). Again, the expression profiles of these three genes after normalized by the VSN algorithm (Figure S1B) are well corresponding to the previous results of their expression patterns. Classification according to GO terms showed that this subnetwork was significantly enriched with fatty acid and several metabolic related terms, including “phospholipid metabolic process”, “lipid metabolic process”, “3-oxoacyl-[acyl-carrier-protein] reductase (NADPH) activity”, and “phospholipase A2 activity” (Figure 4B and 4C, Table S30). This is clearly consistent with previous knowledge of sporopollenin biochemistry [35–37], indicating that this co-expression network reflects the underlying molecular mechanism for exine formation.

In addition to the above three guide genes, this network contained several fatty acid metabolic genes, such as OsMS2, OsAOS5 (ACIL-COA SYNTHETASES), OsPKs (POLYKETIDE SYNTHASE), and OsTKPRs (TETRAKETIDE a-PYRONE REDUCTASES), homologs of which have been demonstrated to be important for sporopollenin biosynthesis in Arabidopsis [41–44] (Figure 4A; Table 2). In the latest model of the sporopollenin biosynthetic pathway [44,45], fatty acid precursors are esterified to CoA by ACOS5, and then hydroxylated by CYP703A and CYP704B to produce substrates of the subsequent ACOS5 reaction (Figure 5). Subsequently, ACOS5, PKs, TKPRs, and MS2 mediate the biochemical reactions to produce sporopollenin precursors via fatty alcohols (Figure 5). Consistently, our pollen wall specific network confirmed significant gene interactions between these molecular players (red bold lines in Figure 4A). Furthermore, in order to identify an as-yet-unknown specific thioesterase producing the CYP703A/CYP704B substrates, we compared the expression patterns of 15 rice thioesterase genes with those of the sporopollenin biosynthetic genes, and detected one thioesterase gene (Os09g0517700) that was co-expressed with OsPKS and OsTKPR2 at the significant PCC level (Table 3). Although Os09g0517700 was not found on the pollen wall synthesis subnetwork (Figure 4A) because of higher MR values, it might play an important role in the sporopollenin biosynthetic pathway.

In addition to the previously characterized genes, several novel genes, whose products participate in fatty acid metabolism, secondary metabolism, and lipid transport, were identified within this network (Figure 4A and Table 2). It is probable that these genes are associated with novel metabolic processes related to sporopollenin biosynthesis or sporopollenin transfer from tapetal cells to anther locules (Figure 5).

It has also been reported that some MYB and bHLH transcriptional factors, including GAMYB, AMS, UDT1, and AtMYB103, regulate the expression of sporopollenin biosynthetic genes [38,46–48]. The 108 co-expressed genes in the pollen wall synthesis network included the two candidates (Os01g0293100 and Os03g0296000) for the transcriptional regulation for sporopollenin biosynthesis network included the two candidates (Os01g0293100 and Os03g0296000) for the transcriptional regulation for sporopollenin biosynthesis (Figure 4A and Table 2). Os03g0296000 is a rice homolog of Arabidopsis MIBB5 (TDF1) [49] responsible for tapetum development, whereas Os01g0293100 is a novel bHLH type transcription factor, not previously studied. In this network, Os01g0293100 is directly connected to CYP704B2 (blue line in Figure 4A), suggesting that Os01g0293100 could be involved in sporopollenin biosynthesis via modulating CYP704B2 expression (Figure 5). Thus, the pollen wall synthesis network in the present co-expression analysis efficiently provides us with useful information to identify novel gene functions and interactions.
Discussion

Co-expression network analysis in plants

In Arabidopsis, useful databases for co-expression analysis are being constructed, and several novel genes have been identified from these data. Aoki et al. [3] and Obayashi et al. [50] listed the available co-expression databases in Arabidopsis, including CressExpress [51], CoP [52], ACT [53], GeneCAT [54], and ATTED-II [55]. As described in these review articles, novel genes for enzymes involved in metabolic pathways, signal factors of biological events (including transcription factors), and components of protein complexes have been identified by using the publicly available databases or analyzing a laboratory’s own co-expression networks. For example, irregular xylem8 (IRX8) and irregular xylem13 (IRX13), which are involved in secondary cell wall formation, were identified from co-expression analysis guided by members of a cellulose synthase (CESA) family [4].

Table 1. Known or putative meiotic genes in the meiosis-specific subnetwork.

| RAP-ID       | Gene_name | Description                          | Category     |
|--------------|-----------|--------------------------------------|--------------|
| Os03g0800200 | MEL1      | PAZ domain containing protein        | Guide gene   |
| Os12g0572800 | MEL2      | RNA recognition motif family protein  | Known meiotic gene |
| Os01g0907900 | OsAML1    | AML1, putative, expressed             | Known meiotic gene |
| Os05g0129900 | RNA polymerase | RNA polymerase II-associated protein 3 | Putative    |
| Os08g0399500 | SET domain | YDG/SRA domain containing protein   | Putative     |
| Os05g0222500 | PWWP domain | PWWP domain containing protein      | Putative     |
| Os08g0384100 | Double-stranded RNA binding | double-stranded RNA binding motif containing protein | Putative |
| Os02g0730100 | Ribonucleoprotein | pre-mRNA processing ribonucleoprotein, binding region | Putative |

Homologous pairing

| RAP-ID       | Gene_name | Description                          | Category     |
|--------------|-----------|--------------------------------------|--------------|
| Os03g0106300 | PAIR1     | PAIR1                                | Guide gene   |

Synapsis

| RAP-ID       | Gene_name | Description                          | Category     |
|--------------|-----------|--------------------------------------|--------------|
| Os09g0506800 | PAIR2     | retrotransposon protein, putative, SINE subclass | Guide gene   |
| Os04g0452500 | ZEP1      | synaptosomal complex protein 2        | Known meiotic gene |
| Os11g0405500 | PAIR3     | expressed protein                     | Guide gene   |

Meiotic replication and chromosome structure control

| RAP-ID       | Gene_name | Description                          | Category     |
|--------------|-----------|--------------------------------------|--------------|
| Os02g0511900 | OsPOLE1   | POLE1 - Putative DNA polymerase epsilon catalytic subunit | Putative |
| Os09g0521900 | Flap endonuclease | flap endonuclease                 | Putative    |
| Os05g0580500 | OsRAD21-4 | Rad21 / Rec8 like protein, putative  | Guide gene   |
| Os10g0094000 | OsSMC2    | chromosome segregation protein        | Putative     |
| Os06g0693300 | OsRPA2C   | RPA2C                                | Putative     |

Meiotic recombination

| RAP-ID       | Gene_name | Description                          | Category     |
|--------------|-----------|--------------------------------------|--------------|
| Os12g0143800 | DMC1A     | DNA repair protein Rad51              | Guide gene   |
| Os11g0146800 | DMC1B     | DNA repair protein Rad51              | Guide gene   |
| Os04g0648500 | BRCA1-associated protein | BRCA1-associated protein | Putative |
| Os03g0242100 | OsRAD17   | cell cycle checkpoint protein RAD17   | Putative     |
| Os05g0389800 | RNA helicase | ATP-dependent RNA helicase           | Putative     |
| Os07g0636200 | SNF2      | SNF2 family N-terminal domain containing protein | Putative |
| Os02g0617500 | OsMER3    | DEAD/DEAH box helicase domain containing protein | Guide gene |
| Os07g0486000 | OsMSH4    | mutS family domain IV containing protein | Known meiotic gene |
| Os05g0498300 | OsMSH5    | mutS domain V family protein          | Known meiotic gene |

Meiotic progression

| RAP-ID       | Gene_name | Description                          | Category     |
|--------------|-----------|--------------------------------------|--------------|
| Os03g0225200 | OsSDS     | cyclin                               | Guide gene   |
| Os01g0233500 | CycA (Cyclin A) | cyclin-A1                       | Putative     |
| Os01g0281200 | CycB (Cyclin B) | cyclin                           | Putative     |
| Os07g0556000 | CycC (Cyclin D) | cyclin                           | Putative     |
| Os01g0935300 | CUL1      | cullin-1                             | Putative     |
| Os07g0624900 | SKP1B     | SKP1-like protein 1B                 | Putative     |
| Os03g0716200 | OsMMD1    | PHD-finger domain containing protein | Known meiotic gene |
| Os09g0242300 | OsCDC20   | WD domain, G-beta repeat domain containing protein | Putative |

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encodes an unknown protein similar to an arabinogalactan protein. Involvement of IRX8 and IRX13 in secondary cell wall formation was demonstrated by T-DNA insertion mutagenesis. Similarly, Oikawa and co-workers [56] conducted a comparative co-expression analysis between Arabidopsis and rice, and proposed a model for secondary cell wall formation of Arabidopsis and rice, based on the combined approach of co-expression and predicted cellular localization. More recently, by using Arabidopsis co-expression networks, UGP3, a gene coding for UDP-glucose pyrophosphorylase 3 implicated in sulfolipid biosynthesis [57], NAI2 implicated in endoplasmic reticulum body formation [58], BTS, a BRUTUS gene coding for bHLH-type transcription factor

Table 2. Known or putative rice genes involved in pollen wall synthesis in the pollen wall synthesis subnetwork.

| RAP-ID | Gene name | Description | Category |
|--------|-----------|-------------|----------|
| The synthesis of Fatty acids precursors |
| Os12g0242700 | KAR | 3-oxoacyl-reductase, chloroplast precursor | Putative |
| Os02g0504800 | Os02g0504800 | PE-PGRS family protein | Putative |
| Sporopollenin biosynthesis |
| Os04g0310800 | OsACOS5 | AMP-binding domain containing protein | Known pollen wall synthesis gene |
| Os08g0131100 | CYP703A3 | cytochrome P450 | Guide gene |
| Os03g0168600 | CYP704B2 | cytochrome P450 | Guide gene |
| Os10g0484800 | OsPKSA | stilbene synthase | Known pollen wall synthesis gene |
| Os07g0411300 | OsPKSB | chalcone and stilbene synthases | Known pollen wall synthesis gene |
| Os08g0519500 | OsTKPR1 | dihydroflavonol-4-reductase | Known pollen wall synthesis gene |
| Os10g1027500 | OsTKPR2 | dihydroflavonol-4-reductase | Known pollen wall synthesis gene |
| Os03g1067600 | OsMS2 | male sterility protein | Known pollen wall synthesis gene |
| Os090517700 | Os090517700 | acyl-coenzyme A thioesterase 10, mitochondrial precursor | Putative |
| The transcriptional regulation of sporopollenin biosynthetic genes |
| Os10g0293100 | bHLH | helix-loop-helix DNA-binding domain containing protein | Putative |
| Os30g0296000 | OsTDF1 | MYB family transcription factor | Known pollen wall synthesis gene |
| The secondary metabolism |
| Os10g0939300 | Os10g0939300 | glutathione S-transferase | Putative |
| Os10g0651500 | Os10g0651500 | wax synthase isomerase 3 | Putative |
| Os03g0263600 | Os03g0263600 | strictosidine synthase | Putative |
| Os05g0498200 | Os05g0498200 | glycine-rich cell wall protein | Putative |
| Os04g0449800 | Os04g0449800 | alpha/beta hydrolase fold | Putative |
| The transport of Sporopollenin |
| Os11g0582500 | OsC6 | LTP/LPL6 - Protease inhibitor/seed storage/LTP family protein precursor | Guide gene |
| Os07g0244900 | LTP | LTP/LPL1 - Protease inhibitor/seed storage/LTP family protein precursor | Putative |
| Os09g0525300 | LTP | LTP/LPL4 - Protease inhibitor/seed storage/LTP family protein precursor | Putative |
| Os03g0357500 | LTP | LTP/LPL1 - Protease inhibitor/seed storage/LTP family protein precursor | Putative |
| Os08g0546300 | LTP | LTP/LPL4 - Protease inhibitor/seed storage/LTP family protein precursor | Putative |
| Os08g0545800 | LTP | LTP/LPL9 - Protease inhibitor/seed storage/LTP family protein precursor | Putative |
| Os10g0691300 | LTP | LTP/LPL150 - Protease inhibitor/seed storage/LTP family protein precursor | Putative |
| Os08g0496800 | OsRAFTIN | BURP domain containing protein | Known pollen wall synthesis gene |
| Os09g0480900 | BURP | BURP domain containing protein | Putative |
| Os06g0607700 | OsWBC27 | ABC-2 type transporter domain containing protein | Known pollen wall synthesis gene |
Figure 5. The current model of the sporopollenin biosynthetic pathway. doi:10.1371/journal.pone.0026162.g005

co-expressed with POPEYE in iron homeostasis [59], and STOMAGEN, encoding secretory peptide regulating stomatal density [60], were successfully identified. Thus, co-expression network analysis is a powerful tool for the identification of novel Arabidopsis genes, and such an approach is undoubtedly also applicable to rice research.

In the present study, we performed co-expression network analysis of rice genes by using 176 datasets of 44 K agilent microarrays (including 33 LM-microarray datasets of the male reproductive organs), and constructed two different co-expression subnetworks (meiosis-specific and pollen-wall-synthesis networks) to demonstrate the efficiency of this technique. Integrative application of such comprehensive microarray data enabled us to construct high-resolution networks (Figures 3A and 4A), which included useful information for the dissection of gene regulation in biological processes. This demonstrates that we can now perform co-expression screening to pursue our interests in specific genes or biological processes. This demonstrates that we can now perform co-expression screening to pursue our interests in specific genes or biological processes in rice.

To the best of our knowledge, this is one of the few reports of the application of co-expression network analysis to specific biological processes in rice. Four rice co-expression databases are now publicly available. In the most recent version of ATTED-II, the co-expression network can be constructed by using the mutual rank of the Pearson’s correlation coefficient (PCC) in rice as well as in Arabidopsis [61]. Ficklin et al. [62] demonstrated application of the Gene Co-expression Network Browser, a website providing a rice co-expression network constructed by random matrix theory and weighted correlation network analysis. In addition, RiceArrayNet [63], based on PCC, and OryzaExpress [64], based on correspondence analysis, are available as rice co-expression databases. These four recent papers [61–64] reported the availability of the rice co-expression databases, but did not provide a demonstration of the identification of genes in relation to specific biological events. Therefore, our present report is a valuable addition to the understanding of the utility of rice co-expression network analysis, together with discussion of specific examples of successful construction of meiosis-specific and pollen-wall-synthesis networks.

Application of co-expression network analysis in sexual reproduction research

Our co-expression analysis included the LM microarray data of male reproductive organs, which can discriminate transcriptomes of gametophytic pollen/microspore cells and sporophytic tapetum cells in the anther, and will provide more precise information on transcriptional regulation of anther genes than has been available previously. None of the examples of gene identification using Arabidopsis co-expression networks described above focus on the microarray databases of reproductive organs, conditions or developmental stages [4,56–60]. Hence, our co-expression network datasets, focusing on male gametophyte development, shed light on a new and interesting aspect of the potential of co-expression analysis. It may be interesting to apply co-expression network analysis to other LM microarray datasets targeted at specific tissues and cells.

Our meiosis-specific and pollen-wall-synthesis networks reveal that many previously unidentified genes are co-expressed in these specific reproductive events. A reverse genetics approach to the study of these genes will lead to identification of novel meiosis-specific and pollen-wall-synthesis genes. Because there are many interesting and specific biological events during male gametophyte development, the very large co-expression datasets presented here will provide an excellent opportunity for plant researchers to study the molecular biology of sexual reproduction in rice.

Materials and Methods

Plant materials

Rice (O. sativa L. ssp. japonica cv. Nipponbare) plants were grown in a greenhouse under normal conditions. Anthers at the premeiosis stage were collected after their developmental stages were confirmed by DAPI staining using one of the six anthers of each flower.

LM

Anthers were fixed in Farmer’s fixative (ethanol: acetate = 3:1) overnight at 4°C. Dehydration and paraffin embedding were performed using a microwave processor [31]. Paraaffin-embedded sections were cut to a thickness of 16 µm and mounted on PEN membrane glass slides (Molecular Devices, Sunnyvale, CA, USA) for LM. To remove paraffin, slides were immersed in 100% xylene (twice), 50% xylene/50% ethanol, and 100% ethanol (v/v), for 5 min at each step and then air-dried completely at room temperature. Three or four individual flowers were used for each LM experiment. LM was performed using the Veritas Laser Microdissection System LCC1704 (Molecular Devices). Selected areas were captured by an infrared (IR) laser onto CapSure Macro
LCM Caps (Molecular Devices) and subsequently cut by a UV laser. The target cells that fused to the LCM cap were collected by removing the cap from the tissue section.

Microarray analysis
Total RNAs were extracted from the LM cells with a PicoPureTM RNA isolation kit (Molecular Devices), quantified with a Quant-itTM Ribogreen RNA reagent and kit (Invitrogen, Carlsbad, CA, USA), and subjected to the rice 44 K oligo microarray (Agilent Technologies, Santa Clara, CA, USA), which contains ~42,000 oligonucleotides based on the nucleotide sequence and full-length cDNA of the Rice Annotation Project Database (RAP-DB) [65]. Fluorescent probe labeling, hybridization, and scanning were performed according to the manufacturer’s instructions (Agilent Technologies), with slight modifications. Each experiment was performed three times using independently isolated samples (three biological replicates). Feature extraction software (Agilent Technologies) was used to delineate and measure the signal intensity of each spot in the array. All microarray data, including our microarray data and publically available data, were statistically normalized by variance stabilization normalization (VSN) using R software (http://www.r-project.org/). The normalized signal intensities were then transformed to log base 2. All microarray data is MIAME compliant and the raw data from this study have been deposited in Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE29217.

GO and EC analyses
GO terms were obtained for rice genes to classify their biological functions [66]. GO terms for GO enrichment analysis and EC analysis were retrieved from the RAP-DB. EC analysis was performed as previously described [21,67], with the following modifications: genes with the same GO term were used as a set of predefined functional genes (each set contained 10 or more genes). EC reports the fraction of gene pairs per GO category that show elevated co-expression. Here, the PCC was utilized as a measure for expression similarity, and we used a PCC threshold of 0.64, corresponding to the 99th percentile of random PCC distribution derived for 1,000 random genes (approximately 1,000 * 990.5 gene pairs). To calculate the random EC for GO categories, random gene sets were sampled with the same size as the category under investigation. For GO enrichment analysis, the statistical significance of GO enrichment within co-expressed genes was evaluated against a background set consisting of 11,456 genes with at least one connection from the dataset of rice co-expression networks using the hypergeometric distribution without a multiple-testing correlation, and P values <0.05 were set as the significant threshold.

Co-expression analysis
To construct co-expression networks, we calculated the PCC values for all combinations of unique 29,864 probes present on the rice 44 K oligo microarray. We estimated two PCC thresholds of 0.48 and 0.65, corresponding to the 95th and 99th percentile of random PCC distribution derived for 1,000 random genes (approximately 1,000 * 990.5 gene pairs). In some previous co-expression studies [3,68], any two genes with a PCC value greater than 0.6 between their expression profiles were considered as co-expressed genes. Therefore, we set a PCC threshold of 0.64 corresponding to the 99th percentile of random PCC distribution, as described above. For all gene pairs with a significant PCC value, we also calculated MR values between them as another value of co-expression measure to further reduce the number of false positives, according to a previous report [69]. Finally, if the absolute value of MR was lower than 10, the gene pair was considered as a significant connection for the co-expression network. These calculations were conducted using the R/Bioconductor. In extracting meiosis or pollen-wall subnetwork datasets, the network vicinity was extracted by taking 2 steps out from a guide gene, as described previously by Mutwil et al. [70]. The network was illustrated using the program Cytoscape.

Table 3. Expression similarities between 15 putative thioesterase genes and 7 sporopollenin biosynthetic genes in rice.

| RAP-ID       | OsTKPR2 | CYP704B2 | OsACOS5 | OsPKSB | CYP703A3 | OsTKPR1 | OsPKSA |
|--------------|---------|----------|---------|--------|----------|---------|--------|
| Os01g0229500 | 0.025   | −0.136   | 0.090   | −0.067 | −0.090   | −0.038  | 0.076  |
| Os01g0229600 | −0.359  | −0.558   | −0.397  | −0.449 | −0.486   | −0.399  | −0.406 |
| Os01g0882000 | −0.098  | 0.174    | −0.005  | −0.076 | −0.027   | −0.019  | −0.018 |
| Os01g0882100 | −0.041  | 0.156    | −0.013  | 0.008  | 0.107    | −0.069  | 0.041  |
| Os02g0521700 | 0.041   | −0.085   | 0.043   | 0.072  | 0.064    | 0.030   | 0.027  |
| Os03g06691400| −0.462  | −0.414   | −0.480  | −0.481 | −0.512   | −0.398  | −0.478 |
| Os04g0436100 | −0.230  | −0.380   | −0.274  | −0.311 | −0.394   | −0.197  | −0.264 |
| Os04g0553300 | 0.375   | 0.601    | 0.403   | 0.439  | 0.533    | 0.336   | 0.431  |
| Os04g0558400 | −0.252  | −0.209   | −0.293  | −0.304 | −0.214   | −0.278  | −0.234 |
| Os05g0137700 | 0.315   | 0.039    | 0.286   | 0.282  | 0.118    | 0.319   | 0.110  |
| Os07g0462700 | −0.087  | −0.395   | −0.119  | −0.170 | −0.330   | −0.060  | −0.191 |
| Os07g0463500 | 0.010   | 0.005    | −0.048  | −0.093 | 0.081    | 0.001   | 0.102  |
| Os09g0517700 | 0.672*  | 0.242    | 0.584   | 0.664* | 0.541    | 0.598   | 0.460  |
| Os02g0660800 | N.T.    | N.T.     | N.T.    | N.T.   | N.T.     | N.T.    | N.T.   |
| Os04g0553100 | N.T.    | N.T.     | N.T.    | N.T.   | N.T.     | N.T.    | N.T.   |

The PCC value of each gene pair was used as a measure of expression similarity. Values with asterisk indicate a higher PCC value than the threshold value, 0.64. N.T., Not tested because of no probe in microarray slide.
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Supporting Information

Figure S1  Representative tissue expression profile of the guide genes after normalization by VSN algorism. Nine meiosis guide genes (A) are preferentially expressed in anther and microspore at meiosis (MEI) stage, whereas three pollen wall guide genes (B) are expressed in microspore and tapetum at tetrad (TET) or uninuclear (UNI) stage. The bar at top represents the average signal intensity (log2). Details on the individual samples can be found in Table S1. All 176 microarray data used for co-expression analysis show a similar expression pattern to those of these guide genes, respectively (Tables S2, S3, S4, S5, S6, S7, S8, S9, S10, S11, S12, S13, S14, S15, S16, S17, S18, S19, S20, S21, S22, S23, S24, S25).

Table S1  GEO IDs of the 176 microarray profiles in this study.

Table S2  Microarray data of Leaf Blade preferentially expressed genes.

Table S3  Microarray data of Leaf Sheath preferentially expressed genes.

Table S4  Microarray data of Root preferentially expressed genes.

Table S5  Microarray data of Stem preferentially expressed genes.

Table S6  Microarray data of Inflorescence preferentially expressed genes.

Table S7  Microarray data of Anther_0.3–0.6 mm preferentially expressed genes.

Table S8  Microarray data of Premeiosis anther preferentially expressed genes.

Table S9  Microarray data of Meiosis (MEI)-microspore preferentially expressed genes.

Table S10  Microarray data of Meiosis (MEI)-tapetum preferentially expressed genes.

Table S11  Microarray data of Anther_0.7–1.0 mm preferentially expressed genes.

Table S12  Microarray data of Tetrad (TET)-microspore preferentially expressed genes.

Table S13  Microarray data of Tetrad (TET)-tapetum preferentially expressed genes.

Table S14  Microarray data of Anther_1.2–1.5 mm preferentially expressed genes.

Table S15  Microarray data of Uninuclear (UN)-microspore preferentially expressed genes.

Table S16  Microarray data of Uninuclear (UN)-tapetum preferentially expressed genes.

Table S17  Microarray data of Anther_1.6–2.0 mm preferentially expressed genes.

Table S18  Microarray data of Bicellular (BC)-pollen preferentially expressed genes.

Table S19  Microarray data of Tricellular (TC)-pollen preferentially expressed genes.

Table S20  Microarray data of Pistil preferentially expressed genes.

Table S21  Microarray data of Lemma preferentially expressed genes.

Table S22  Microarray data of Palea preferentially expressed genes.

Table S23  Microarray data of Ovary preferentially expressed genes.

Table S24  Microarray data of Embryo preferentially expressed genes.

Table S25  Microarray data of Endosperm preferentially expressed genes.

Table S26  EC scores for different GO categories.

Table S27  PCC and MR values of all gene pairs in the meiotic subnetwork.

Table S28  Frequency of GO categories for genes in the meiotic subnetwork.

Table S29  PCC and MR values of all gene pairs in the pollen wall synthesis subnetwork.

Table S30  Frequency of GO categories for genes in the pollen wall synthesis subnetwork.

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Author Contributions
Conceived and directed the project: MW MM. Designed and performed laser microdissection experiment: HT K. Shiiono NT MN. Designed and performed microarray analysis: YN. Analyzed microarray data and performed GO, EC and co-expression network analyses: KA GS K. Swuabe KH KY TH. Discussed the results and commented on the manuscript: KA GS K. Swuabe TH HT K. Shiiono KY NT MN YN MW. Wrote the manuscript: KA GS K. Swuabe MW MM.

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Network Analysis of Anther-Expressed Genes in Rice
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