



# Rice *ORMDL* Controls Sphingolipid Homeostasis Affecting Fertility Resulting from Abnormal Pollen Development

Chutharat Chueasiri<sup>1</sup>, Ketsuwan Chunthong<sup>1</sup>, Keasinee Pitnjam<sup>1</sup>, Sriprapai Chakhonkaen<sup>1</sup>, Numphet Sangarwut<sup>1</sup>, Kanidta Sangsawang<sup>1</sup>, Malinee Suksangpanomrung<sup>1</sup>, Louise V. Michaelson<sup>2</sup>, Johnathan A. Napier<sup>2</sup>, Amorntip Muangprom<sup>1\*</sup>

<sup>1</sup> National Center for Genetic Engineering and Biotechnology, Thailand Science Park, Klong Luang, Pathumthani, Thailand, <sup>2</sup> Biological Chemistry Department, Rothamsted Research, Harpenden, Hertfordshire, United Kingdom

## Abstract

The orosomucoids (ORM) are ER-resident polypeptides encoded by *ORM* and *ORMDL* (ORM-like) genes. In humans, *ORMDL3* was reported as genetic risk factor associated to asthma. In yeast, ORM proteins act as negative regulators of sphingolipid synthesis. Sphingolipids are important molecules regulating several processes including stress responses and apoptosis. However, the function of *ORM/ORMDL* genes in plants has not yet been reported. Previously, we found that temperature sensitive genetic male sterility (TGMS) rice lines controlled by *tms2* contain a deletion of about 70 kb in chromosome 7. We identified four genes expressed in panicles, including an *ORMDL* ortholog, as candidates for *tms2*. In this report, we quantified expression of the only two candidate genes normally expressed in anthers of wild type plants grown in controlled growth rooms for fertile and sterile conditions. We found that only the *ORMDL* gene (*LOC\_Os07g26940*) showed differential expression under these conditions. To better understand the function of rice *ORMDL* genes, we generated RNAi transgenic rice plants suppressing either *LOC\_Os07g26940*, or all three *ORMDL* genes present in rice. We found that the RNAi transgenic plants with low expression of either *LOC\_Os07g26940* alone or all three *ORMDL* genes were sterile, having abnormal pollen morphology and staining. In addition, we found that both sphingolipid metabolism and expression of genes involved in sphingolipid synthesis were perturbed in the *tms2* mutant, analogous to the role of ORMs in yeast. Our results indicated that plant *ORMDL* proteins influence sphingolipid homeostasis, and deletion of this gene affected fertility resulting from abnormal pollen development.

**Citation:** Chueasiri C, Chunthong K, Pitnjam K, Chakhonkaen S, Sangarwut N, et al. (2014) Rice *ORMDL* Controls Sphingolipid Homeostasis Affecting Fertility Resulting from Abnormal Pollen Development. PLoS ONE 9(9): e106386. doi:10.1371/journal.pone.0106386

**Editor:** Carl Ng, University College Dublin, Ireland

**Received:** September 28, 2012; **Accepted:** August 7, 2014; **Published:** September 5, 2014

**Copyright:** © 2014 Chueasiri et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

**Funding:** This work was supported by National Science and Technology Development Agency; grant numbers P-00-20101. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing Interests:** The authors have declared that no competing interests exist.

\* Email: amorntip.mua@biotec.or.th

## Introduction

Orosomucoid (ORM) family proteins are ER proteins encoded by *ORM* and *ORMDL* (ORM-like) genes. These proteins are highly conserved from yeasts to plants to human. Recently, human *ORMDL3* was reported as a genetic risk factor associated with asthma in diverse populations [1–3]. In addition, the expression of *ORMDL3* gene was shown to be associated with the disease symptoms [4,5]. *ORMDL3* is involved in pro-inflammatory diseases by binding and inhibiting sarco-endoplasmic reticulum  $\text{Ca}^{2+}$  pump (SERCA), which reduce ER  $\text{Ca}^{2+}$  concentration and increase unfolded-protein response (UPR), a process believed to induce inflammation [6].

In yeast, deletion of the ORM proteins showed susceptibility to agents that increase protein-misfolding in the ER [7]. In addition, deletion of the ORM proteins increased UPR and slowed ER-to-Golgi transport of the tested proteins though these effects were suppressed by high temperatures [8]. After treating with agents that increase protein-misfolding, *ORM2* gene expression was increased significantly, indicating that it is up-regulated by UPR

[8]. In addition, genetic interaction profiles of *orm2Δ* deletion mutants showed inverse correlation with the interaction patterns of yeast mutants with reduced *LCB1* and *LCB2* expression. Furthermore, yeast cells with over-expression of *ORM1* or *ORM2* showed genetic interaction profiles highly correlated with those seen in yeasts with reduced *LCB1* and *LCB2* expression, indicating that increased expression of ORM reduced LCB 1/2 activity [9]. *LCB1* and *LCB2* encode serine palmitoyltransferase, the first and rate-limiting enzyme in sphingolipid biosynthesis. Over-expression of *ORM1* or *ORM2* resulted in reduced LCB levels, while cells with deleted *ORM1/2* had highly elevated levels of LCBs [9]. Thus, it was proposed that ORM proteins were negative regulators of sphingolipid synthesis [9]. Yeast cells with *ORM1/2* deletions increased flux throughout the sphingolipid pathway, resulting in growth defects [9] though the precise metabolic impact of loss of ORM function on sphingolipid synthesis in yeast is currently ambiguous, since *orm1Δ/orm2Δ* mutants have been reported to have reduced [8] or elevated [9] levels of ceramides. In addition, alteration in *ORM* gene expression or mutations to their phosphorylation sites perturbed

sphingolipid metabolism, leading to the hypothesis that ORMs play a central role in lipid homeostasis [9,10].

Sphingolipids are key cellular membranes and signaling molecules involved in several cellular activities, such as cell proliferation, cell differentiation, apoptosis, and stress responses [11–14]. In plants, sphingolipids are important for cell growth and the establishment of cell polarity through their contribution to the functional organization of the endomembrane system [15]. Accordingly, sphingolipids were reported to be involved in a trafficking pathway with specific endomembrane compartments and polar auxin transport protein [16]. Sphingolipids are also reported to be involved in responses to ABA and cold temperature [17–19], also regulated mineral ion homeostasis and programmed cell death [20,21].

Microsporogenesis has been reported to be controlled by sphingolipids in several plant species [22–24]. In Arabidopsis, the study of *fbr11-2/lcb1-1* mutants showed that the *fbr11-2* mutant, an allele of *lcb1-1*, was transmitted only through female gametophytes, and initiated apoptotic cell death in bi-nucleated microspores [23]. The *FBR11/LCB1* expression was confined in microspores during microgametogenesis. These results suggested that SPT modulated programmed cell death plays an important role in the regulation of male gametophyte development [23]. In addition, Arabidopsis *LCB2* loss-of-function mutant demonstrated that sphingolipids are important for gametophytic development by alterations in the endomembrane system of pollen [22]. Furthermore, sterile or severely reduced in fertility were observed in antisense or RNAi transgenic plants with repressed expression of *dihydrosphingosine C4 hydroxylase 1 (DSH 1)*, a key constituent of sphingolipids, which supported the role of sphingolipids on fertility in plant [25].

Male sterility facilitates hybrid seed production. Rice hybrids showed 20% higher yields than the best inbred varieties [26]. Hybrid rice is a promising alternative to increase food production to meet the demand for future need. Previously, we found that *temperature-sensitive genetic male-sterile 2 (tms2)* rice line (TGMS) lacked a 70Kb section of DNA that encoded 4 expressed genes, one of which is an *ORMDL* ortholog [27]. Although there are three *ORMDL* genes in rice, only one (*LOC\_Os07g26940*) is located in the deleted region. In this report, we found that the *LOC\_Os07g26940* gene showed strong expression in rice panicles and anthers. In addition, *LOC\_Os07g26940* showed differential expression under low and high temperatures, corresponding to fertile and sterile conditions, respectively. When RNAi was used to suppress *ORMDL* genes, either in the deletion region or all *ORMDL* three genes in rice, the resulting transgenic plants showed abnormal pollen development that resulted in male sterility. In addition, we showed that sphingolipids metabolism and expression of genes in sphingolipids biosynthesis were perturbed in the *tms2* mutants. Similar to yeast, our results indicated that the plant *ORMDL* gene modulates sphingolipid homeostasis, affecting fertility by controlling pollen development. Therefore, understanding the processes that control plant fertility could be useful in generating high yield crops by hybrid or by genetic engineering.

## Results

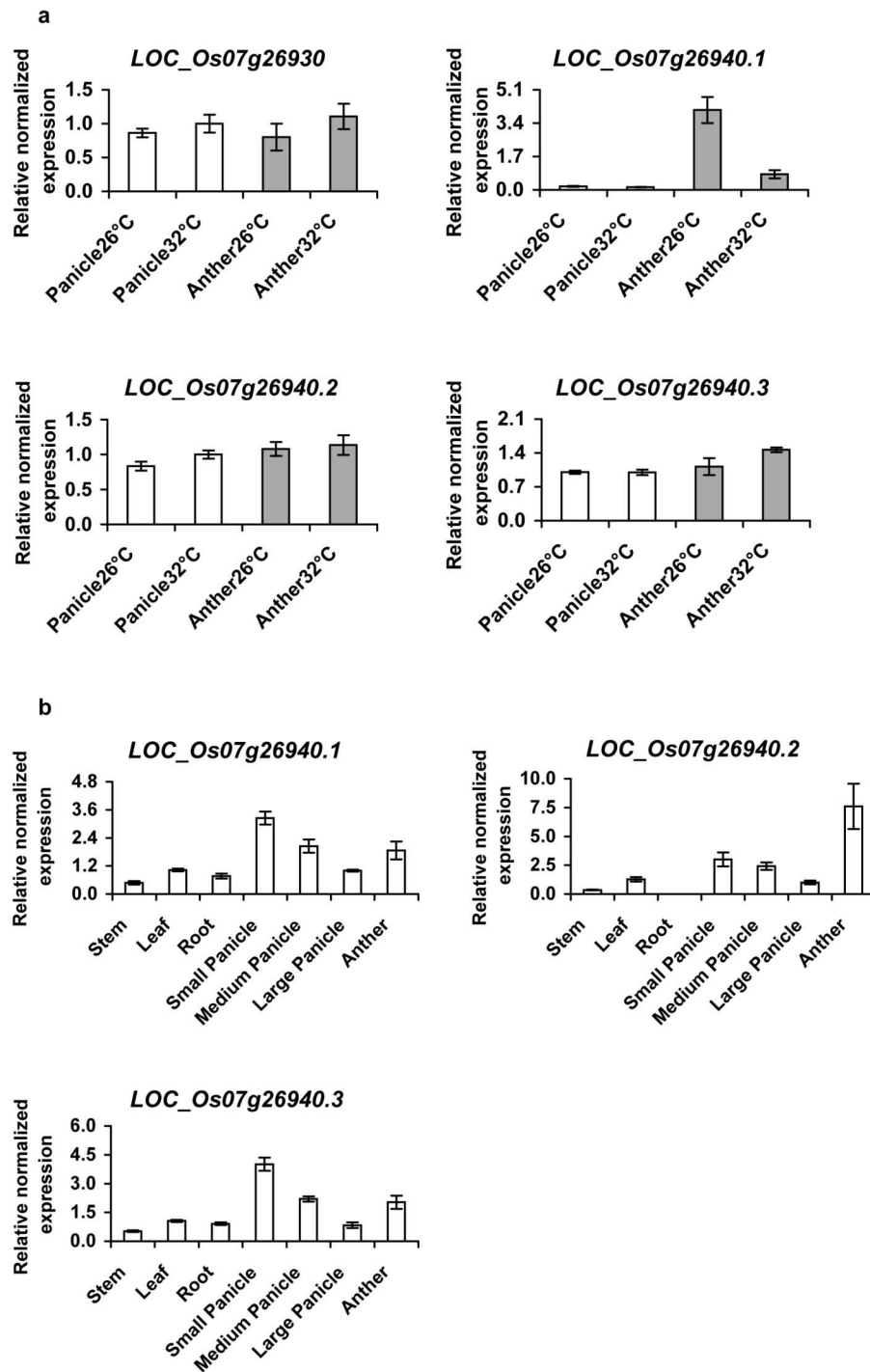
### Expression analysis of candidate genes

Seven genes located in the 70 Kb loci associated with the TGMS *tms2* phenotype were annotated to encode expressed proteins, four of which expressed in panicles [27]. In this study, we examined the expression of these four genes (*putative cytochrome P450 77A3 LOC\_Os07g26870*, *LOC\_Os07g26930*, *ORMDL*

*LOC\_Os07g26940*, and *LOC\_Os 07g26974*) in anthers of wild type plants by RT-PCR, confirming the identity of the amplicons by sequencing. Our results indicated that only *LOC\_Os07g26930* and *ORMDL LOC\_Os07g26940* were expressed in anthers (data not shown). To determine whether *LOC\_Os07g26930* and *ORMDL LOC\_Os07g26940* were differentially expressed at low and high temperatures corresponding to fertile and sterile conditions, the expression of these genes was monitored in panicles and anthers of wild type plants grown in controlled growth room at 26 and 32°C, using qRT-PCR. Information from GRAMENE rice database indicated that *LOC\_Os07g26930* and *ORMDL LOC\_Os07g26940* have 1 and 3 splicing forms respectively. Primers specific for all these forms were designed and used for expression analysis by qRT-PCR, compared to *Actin1* as internal control. The results showed that expression of *LOC\_Os07g26930* was constant at low and high temperatures in panicle or in anther of wild type plants (Fig. 1A), inconsistent with a role in TGMS. In the case of *ORMDL LOC\_Os07g26940*, we detected all three splicing forms by qRT-PCR. The results showed that expression levels of *ORMDL LOC\_Os07g26940.1* was similar at low and high temperatures in the panicle. Interestingly in the anther, this splicing form showed much higher expression at low temperature (fertile condition) than that at high temperature (sterile condition) (Fig. 1A). *ORMDL LOC\_Os07g26940.2* and *ORMDL LOC\_Os07g26940.3* showed similar levels of expression at low and high temperatures in panicle or in anther, with a little higher at high temperatures in panicle for *ORMDL LOC\_Os07g26940.2*. While *ORMDL LOC\_Os07g26940.3* showed a little higher expression in the anther at high temperature (sterile condition) than at low temperature (fertile condition) (Fig. 1A). To further study tissue-specific expression of *ORMDL LOC\_Os07g26940*, multiple different rice tissues were used for qRT-PCR analyses including leaves, stems, roots, anthers, small panicles, medium panicles, and large panicles. Using *elongation factor 1  $\alpha$* , *EF-1 $\alpha$*  as a reference gene, the results indicated that all the three transcripts derived from *ORMDL LOC\_Os 07g26940* were detected in most of the tested tissues, except in root where we could not detect expression of *ORMDL LOC\_Os 07g26940.2*. The three forms were predominantly accumulated in the panicle and anther (Fig. 1B).

### Phylogenetic Analysis

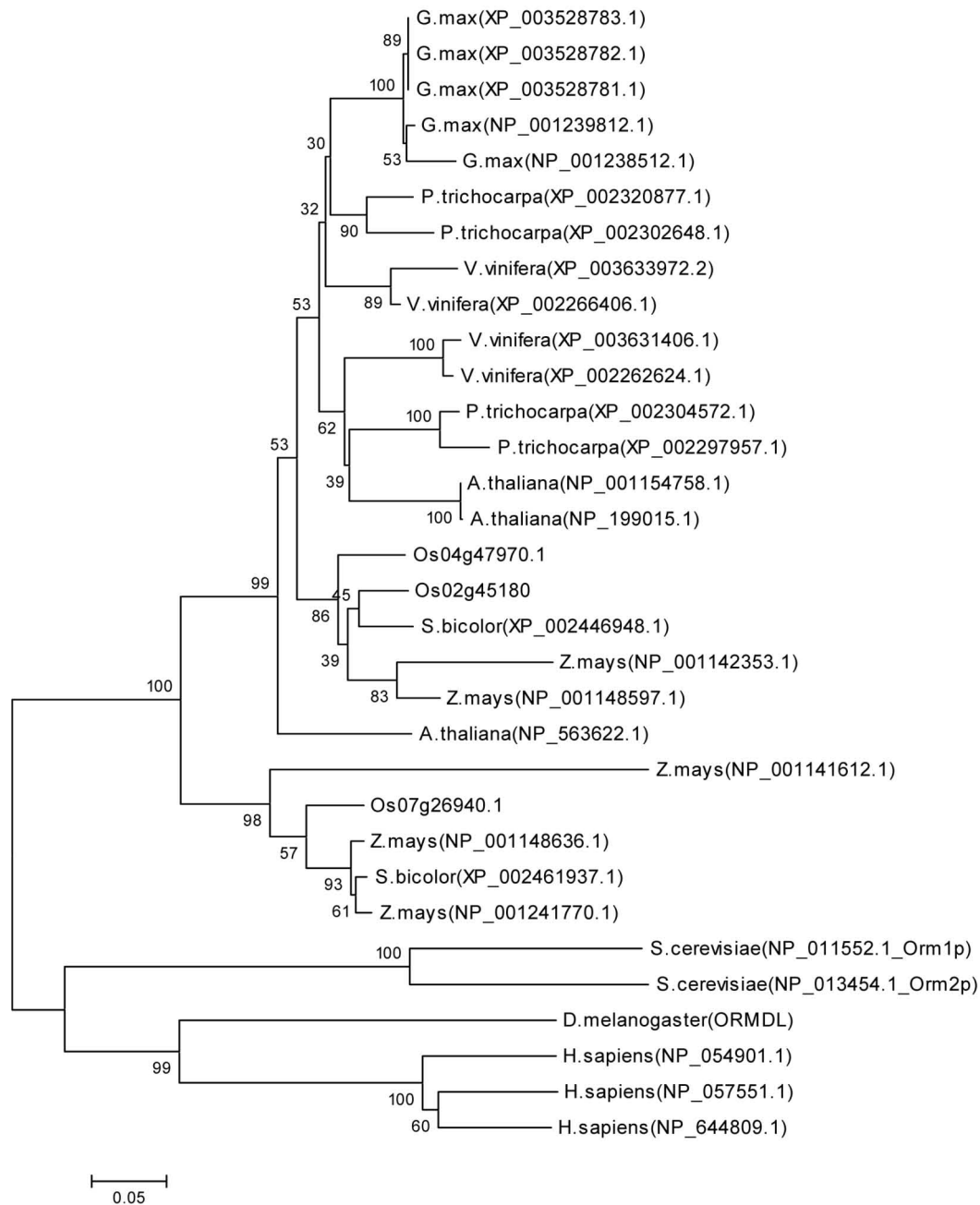
*LOC\_Os07g26940* is a member of the *ORMDL* protein family, which is highly conserved in eukaryotes. Information from the GRAMENE rice database indicated that *ORMDL LOC\_Os07g26940* has three splicing forms, and the results from sequence alignments of these transcripts indicated that the three transcripts of *ORMDL LOC\_Os07g26940* shared the same sequence for about half of the gene starting from the beginning of the transcripts (Fig. S1). Previously, a BLAST search using the predicted amino acid sequences of *ORMDL LOC\_Os07g26940* revealed two other sequences (encoded by *LOC\_Os04g47970* and *LOC\_Os02g45180*) with high percent sequence similarity to *ORMDL LOC\_Os07g26940*, suggesting that at least three *ORMDL* genes are present in rice [27]. *ORMDL LOC\_Os04g47970* has two splicing forms but *ORMDL LOC\_Os02g45180* has only one. Similarly, results from comparing the two transcripts of *ORMDL LOC\_Os04g47970* showed that they share about 75% of the sequences starting from the beginning of the transcripts (Fig. S2). The rice transcripts of *ORMDL* showed high homology in the coding sequences (Fig. S3). To understand the evolutionary relationships of this gene family in rice and other species across kingdoms, phylogenetic analysis was constructed, using deduced amino acid sequences from the longest transcript of



**Figure 1. Expression analysis by quantitative RT-PCR.** (A) qRT-PCR analysis of *LOC\_Os07g26930* and all three splicing forms of *ORMDL* (*LOC\_Os07g26940*) in panicles and anthers of wild type rice plants grown in control growth room at 26°C and 32°C. The expression level of *Actin1* (*Os05g36290*) was used as internal control. (B) qRT-PCR analysis of all three splicing forms of *LOC\_Os07g26940* in wild type rice tissues, including stems, leaves, roots, small panicles (1–2 cm), medium panicles (3–14 cm), large panicles (15–25 cm) and anthers. The expression level of *EF-1 $\alpha$*  (*Os03g08020*) was used as internal control. All data are representative from at least two biological repeats, each based on three technical replicates; similar results were obtained in the repeated experiments. Bars indicate the standard error (n=3), which was calculated from technical replicates. Each biological sample was a mixture of 3 plants.  
doi:10.1371/journal.pone.0106386.g001

each rice *ORMDL*. This analysis showed the phylogenetic relationship between rice *ORMDL* and other *ORMDL* from different species selected from NCBI Reference Sequence database. The results showed 4 main groups of genes from

human, drosophila, yeast, and plants. As expected, rice *ORMDL* is closer to *Z. mays* and to *S. bicolor* than to *A. thaliana*. Interestingly, *ORMDL* *LOC\_Os07g26940* is in a separate clade from the other two rice *ORMDL* (Fig. 2).



**Figure 2. Phylogenetic relationship of rice *ORMDL* and other *ORMDL* from different species across kingdom.** An un-rooted neighbour joining phylogenetic tree was constructed from the protein sequences of rice *ORMDL* obtained from GRAMENE rice database and *ORMDL* from other species selected from NCBI Reference Sequence database. Multiple sequence alignment was performed using the ClustalW in MEGA5 and the tree was generated using MEGA5. The numbers for interior branches indicate the bootstrap values (%) for 1000 replications. doi:10.1371/journal.pone.0106386.g002

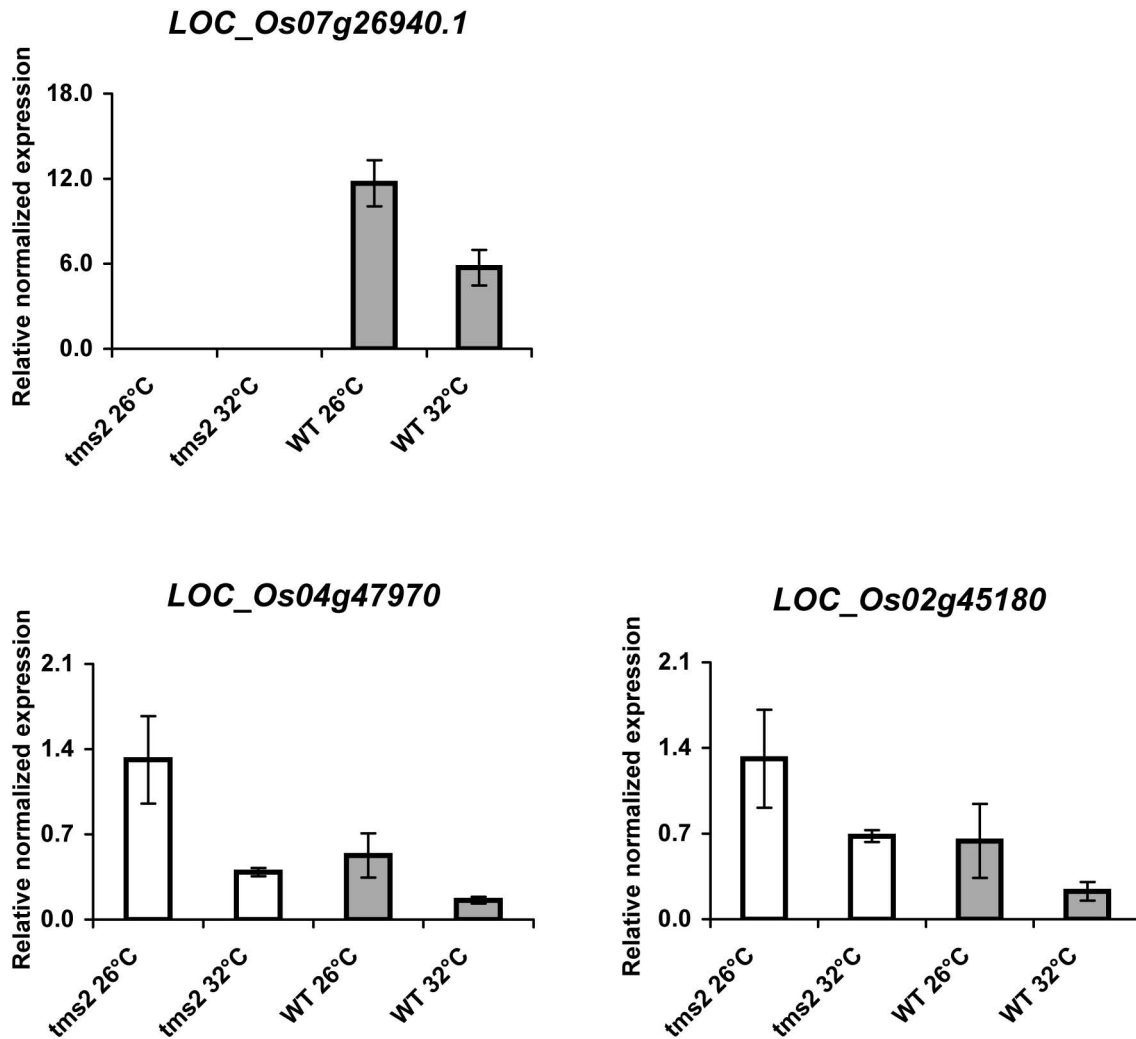
### Expression analysis of rice *ORMDL* genes

To determine the patterns of expressions of rice *ORMDL* genes in *tms2* and wild type rice plants, expression of these genes by qRT-PCR was monitored in anthers of *tms2* and wild type plants grown in controlled growth room at 26 and 32°C. Using *EF-1 $\alpha$*  as the reference gene, the results indicated that all the three *ORMDL* genes in rice showed similar patterns of expression by having higher expression at low temperature (26°C) than that at high temperature (32°C) conditions, and these genes showed higher expression in *tms2* than in wild type plants in both conditions. As

expected, expression of *ORMDL LOC\_Os07g26940.1*, used as a representative splicing form of *ORMDL LOC\_Os07g26940*, was not detected in the *tms2* mutant rice plants (Fig. 3).

### RNAi transgenic rice plants

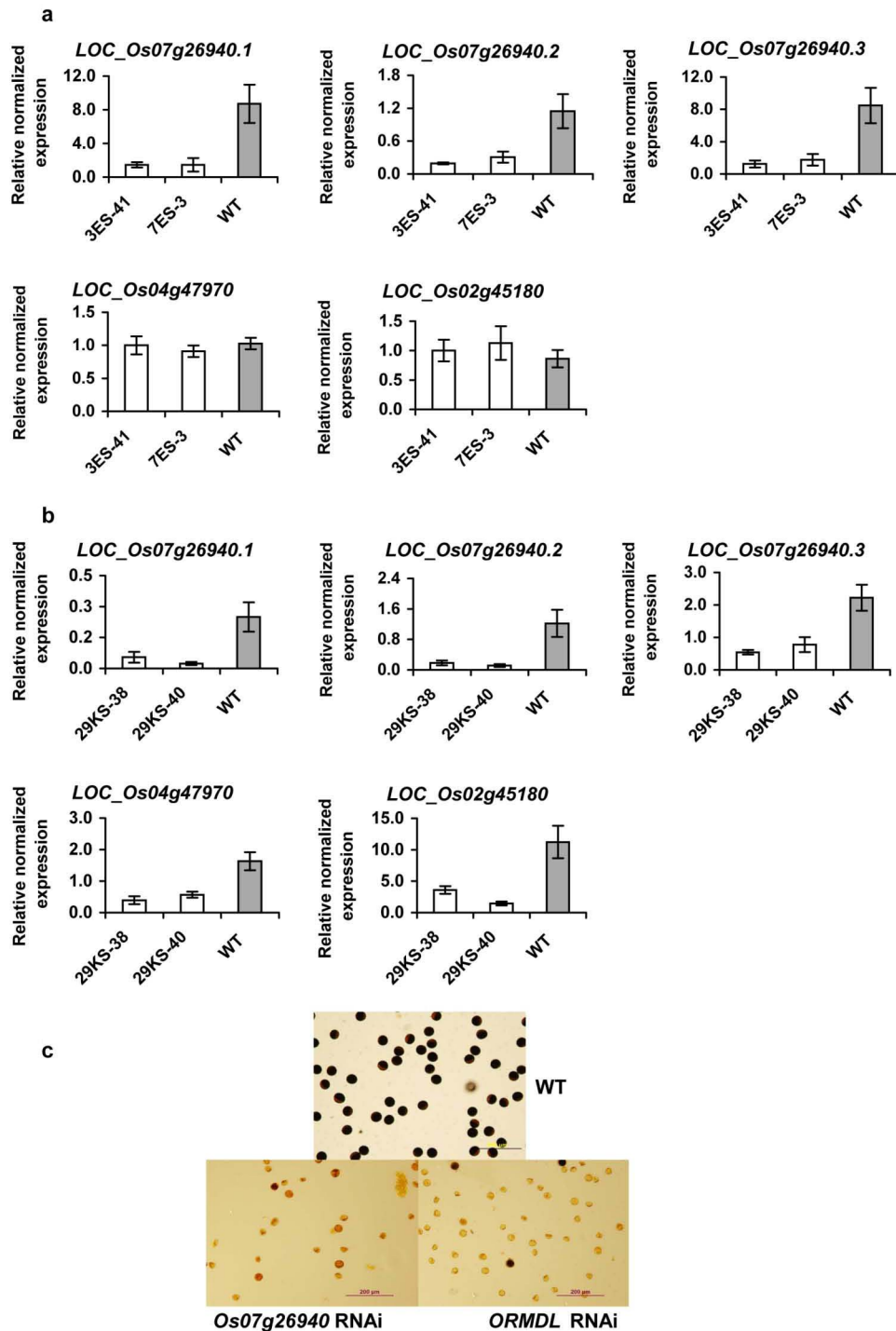
To investigate the role of *LOC\_Os07g26930* and *ORMDL LOC\_Os07g26940* on pollen development, RNAi technology was used to down-regulate *LOC\_Os07g26930* or *ORMDL LOC\_Os07g26940* expression in rice. For the gene-specific *ORMDL LOC\_Os07g26940* RNAi construct, sequence present only in the



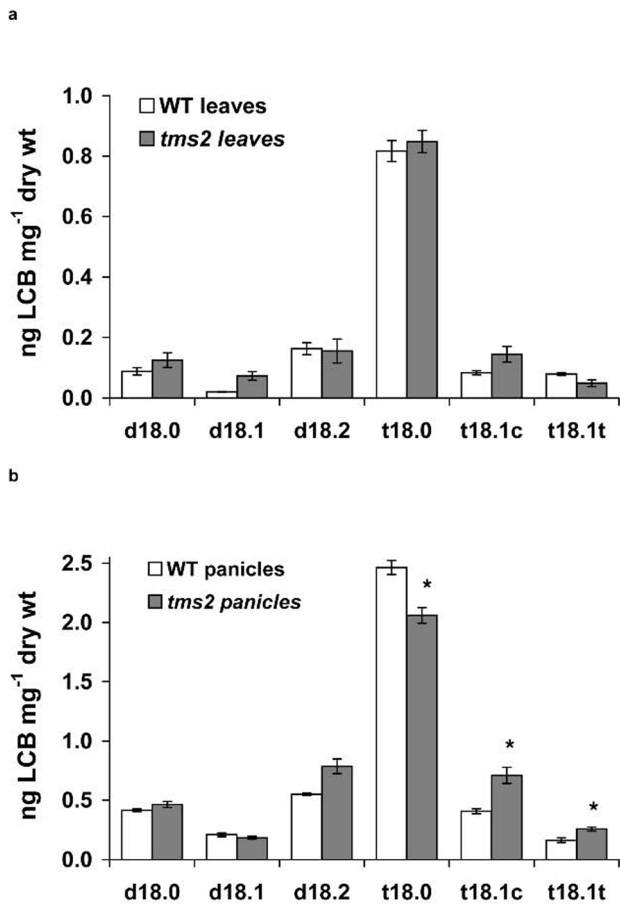
**Figure 3. Expression of rice *ORMDL* genes in *tms2* and wild type rice plants.** qRT-PCR analysis of rice *ORMDL* genes in anthers of *tms2* and wild type rice plants grown in control growth room at 26°C and 32°C. The expression level of *EF-1 $\alpha$*  was used as internal control. All data are representative from at least two biological repeats, each based on three technical replicates; similar results were obtained in the repeated experiments. Bars indicate the standard error (n = 3), which was calculated from technical replicates. Each biological sample was a mixture of 3 plants. doi:10.1371/journal.pone.0106386.g003

transcripts of *ORMDL LOC\_Os07g26940*, but not in *ORMDL* genes (*LOC\_Os04g47970* and *LOC\_Os02g45180*) were used (Fig. S3). As this sequence is different from the other two rice *ORMDL* genes, we predicted that the RNAi only affects *ORMDL LOC\_Os07g26940* expression. However, because three *ORMDL* genes are present in rice, knocking down only the *ORMDL LOC\_Os07g26940* expression may not reveal all phenotypic effects. Thus, we generated another RNAi construct using conserved sequences present in all rice *ORMDL* genes (Fig. S3), to universally down-regulated all the *ORMDL* gene transcripts. These RNAi sequences were under the control of ubiquitin promoter [28]. These RNAi constructs were transformed in to wild type (WT) *Nipponbare* plants. We obtained 27, 8 and 6 transgenic lines for *LOC\_Os07g26930*-specificRNAi, *LOC\_Os07g26940*-specificRNAi, and *ORMDL*-specific RNAi, respectively. In our experience, it was much more difficult to obtain *LOC\_Os07g26940*-specificRNAi, and *ORMDL*-specific RNAi, transgenic lines than that from *LOC\_Os07g26930*-specificRNAi construct. In natural condition, at flowering, none of transgenic plants for *LOC\_Os07g26930*-specificRNAi were sterile. For,

*LOC\_Os07g26940*-specific RNAi, or *ORMDL*-specific RNAi, two independent transformed lines from each construct were selected for further studies on the basis of low levels of expression of the target genes and a single copy of the transgene construct. As expected, expressions of all three splicing forms of *ORMDL LOC\_Os07g26940* were reduced in *LOC\_Os07g26940*-specific RNAi transgenic plants, while expression of the other two *ORMDL* were not lower than that of wild type plants (Fig. 4A). For *ORMDL*-specific RNAi, expressions of all three *ORMDL* were reduced (Figure 4B). At flowering, the pollen of these plants was different from that of wild type rice plants in both morphology and staining. The wild type pollen was round, showing the presence of starch granules, while pollen of *LOC\_Os07g26940*-specificRNAi, or *ORMDL*-specific RNAi, was abnormal showing small pear-shaped pollen grains lacking starch (Fig. 4C). In addition, these T0 transgenic plants were sterile when they were grown under glass house conditions. Thus, they were ratooned from one T0 plant to at least 6 plants from each of the original T0 transgenic plant. The plants were grown under glass house conditions for approximately one month. Subsequently, three



**Figure 4. Expression analysis and pollen staining of RNAi transgenic rice plants.** A) qRT-PCR analysis of *ORMDL* *LOC\_Os07g26940* and other *ORMDL* gene expression in leaves of *LOC\_Os07g26940* RNAi (3ES-41 and 7ES-3). B) qRT-PCR analysis of *ORMDL* *LOC\_Os07g26940* and other *ORMDL* gene expression in leaves of *ORMDL* RNAi (29KS38 and 29KS-40) transgenic rice plants. 3ES-41 and 7ES-3 or 29KS38 and 29KS-40 are two independent transgenic lines from the same construct. The expression level of *EF-1 $\alpha$*  was used as internal control. All data are representative from at least two biological repeats, each based on three technical replicates; similar results were obtained in the repeated experiments. Bars indicate the standard error (n=3), which was calculated from technical replicates. Each biological sample was a mixture of 3 plants. C) Pollen staining of wild type, *LOC\_Os07g26940* RNAi, and *ORMDL* RNAi transgenic rice plants. Similar results were obtained from the two tested transgenic lines from each construct. The pictures are a representative from each construct.  
doi:10.1371/journal.pone.0106386.g004

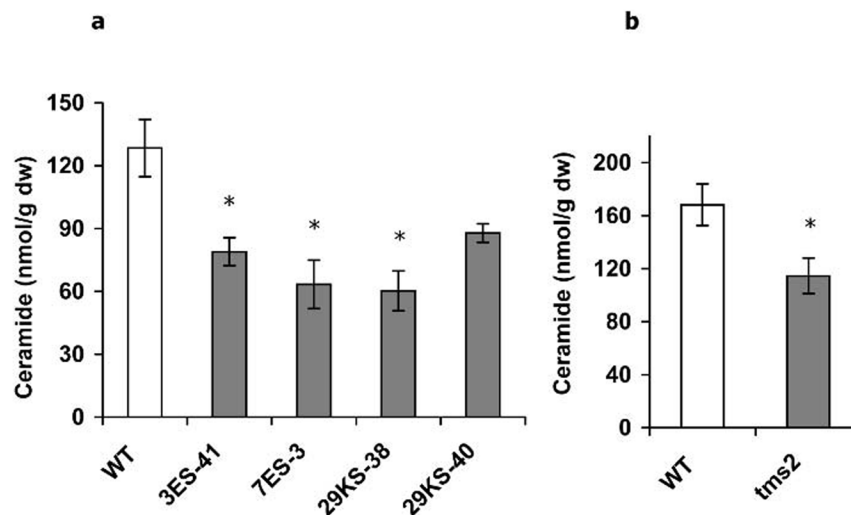


**Figure 5. Sphingolipid long-chain base composition ng LCB mg<sup>-1</sup> dry weight in wild type and *tms2* mutant rice plants in leaf tissue(A) and in panicles(B).**  
doi:10.1371/journal.pone.0106386.g005

plants from each line were moved to grow in control growth room at 26 and 32°C. At flowering, these plants were sterile, with no plants capable of setting seed. The panicle tissue of the T0 was not used for direct measurement as they were retained to observe seed setting and to allow for T1 seed production.

### Sphingolipid metabolism is perturbed in the *tms2* mutant and in RNAi rice lines

Recently, yeast ORM proteins were reported as negative regulators of sphingolipid synthesis [8,9]. Since one *ORMDL* gene was deleted in the rice *tms2* mutant, one scenario is that this may affect or alter sphingolipid synthesis by releasing LCB synthesis from homeostatic regulation. To investigate whether the *tms2* mutant affects sphingolipid composition, total sphingolipid long-chain bases (LCBs) were analyzed from leaves and panicles of wild-type and *tms2* mutant rice plants. Sphingolipids comprise an LCB and an amide-linked a fatty acid moiety, usually also decorated with a sugar head group. The LCB component of sphingolipids is unique to these lipids, and its synthesis in yeast has been shown to be modulated by ORM1/2. Analysis of total LCBs showed that no significant difference in sphingolipid LCB composition was observed in leaves of *tms2* mutant and wild type rice plants (Fig. 5A). Importantly, panicles of *tms2* mutant rice plants had altered sphingolipid LCB composition, with higher levels of d18:2, t18:1c, and t18:1t but lower levels of t18:0, compared with wild type rice plant (Fig. 5B). The other LCB species were similar in *tms2* mutant and wild type rice plants (Fig. 5B). In view of the alteration of LCB composition in the *tms2* mutant, and also previous studies in yeast which revealed altered ceramide levels in *orm1Δorm2Δ* mutants [8,9], more targeted analysis was carried out, to measure the ceramide levels in this mutant and also RNAi-suppressed lines. The results showed that both RNAi-suppressed lines and *tms2* mutant plants showed a reduced accumulation of ceramides, relative to the WT (Fig. 6A–6B; note that in the case of the RNAi lines, analysis was carried out



**Figure 6. Total ceramide analysis.** 6A) Total ceramide analysis in leaves of RNAi rice lines and WT rice plants. 6B) Total ceramide analysis in panicles of *tms2* mutant and WT rice plants. Ceramide levels were determined from the leaves of *LOC\_Os07g26940* RNAi (3ES-41 and 7ES-3) or *ORMDL* RNAi (29KS38 and 29KS-40) transgenic rice plants, and compared with equivalent tissue from WT. Results were expressed as absolute levels of ceramides (nmol/g dw). Similar analysis was performed on panicles of either WT or *tms2* mutant rice, and data is presented as absolute levels of ceramides (nmol/g dw). Absolute levels (A, B) represent the mean of 6 technical replicates for each independent line. Bar indicates the standard error (n=6). \* indicates statistical significance at p-value of 0.05 as determined by t-test. 3ES-41 and 7ES-3 or 29KS38 and 29KS-40 are two independent transgenic lines from the same construct.  
doi:10.1371/journal.pone.0106386.g006



**Table 1.** Ceramide composition in panicles of *Indica* WT and *tms2* rice lines.

	<b>c16:0</b>	<b>c18:0</b>	<b>c20:0</b>	<b>c20:1</b>	<b>c22:0</b>	<b>c22:1</b>	<b>c24:0</b>	<b>c24:1</b>	<b>c26:0</b>	<b>c26:1</b>	
<b>WT</b>	<b>d18:0</b>	0.41±0.09	0.01±0.00	0.13±0.01	0.14±0.02	0.04±0.01	0.02±0.01	0.15±0.01	0.02±0.00	0.23±0.02	0.02±0.01
	<b>d18:1</b>	0.13±0.03	0.03±0.02	0.28±0.09	8.86±1.69	0.23±0.07	1.01±0.10	0.60±0.24	0.19±0.10	0.10±0.03	0.10±0.03
	<b>d18:2</b>	1.61±0.50	0.38±0.03	4.29±0.31	77.85±4.52	0.69±0.07	13.29±1.03	0.52±0.08	0.21±0.07	2.25±0.47	2.25±0.47
	<b>t18:0</b>	0.62±0.06	0.40±0.08	2.52±0.28	0.13±0.02	5.29±0.92	0.46±0.08	18.46±8.21	5.95±2.77	1.39±0.29	1.39±0.29
	<b>t18:1</b>	0.13±0.01	0.07±0.04	0.10±0.01	0.72±0.08	0.15±0.03	1.56±0.11	0.94±0.19	0.51±0.11	0.30±0.05	0.30±0.05
<b>tms</b>	<b>d18:0</b>	0.40±0.19	0.01±0.00	0.07±0.01	0.07±0.03	0.02±0.00	0.01±0.00	0.09±0.02	0.17±0.04	0.01±0.00	0.01±0.00
	<b>d18:1</b>	0.07±0.02	0.01±0.00	0.13±0.03	4.39±1.61	0.16±0.04	0.54±0.13	0.47±0.17	0.19±0.10	0.06±0.10	0.06±0.10
	<b>d18:2</b>	1.59±0.59	0.24±0.06	2.85±0.58	54.81±10.10	0.44±0.09	8.46±1.45	0.35±0.06	0.09±0.02	1.48±0.29	1.48±0.29
	<b>t18:0</b>	0.49±0.10	0.30±0.09	1.31±0.33	0.11±0.03	3.36±0.81	0.25±0.04	13.78±6.70	4.46±2.39	0.64±0.13	0.64±0.13
	<b>t18:1</b>	0.13±0.03	0.02±0.00	0.05±0.01	0.45±0.10	0.13±0.04	0.87±0.20	0.61±0.13	0.33±0.10	0.28±0.07	0.28±0.07

Unit: nmol g dw<sup>-1</sup>, N: a minimum of 3± SE.  
doi:10.1371/journal.pone.0106386.t001

on leaf material). This was true for RNAi which suppressed all rice *ORMDL* transcripts, or specifically just the transcript derived from *LOC\_Os07g26940* and absent in *tms2*. Breakdown of these data is shown in table 1 and 2. These data confirm the specific role for *LOC\_Os07g26940* in modulating ceramide levels in rice.

### Expression of genes in sphingolipid synthesis pathway

Rice *ORMDL* gene *LOC\_Os07g26940* is proposed, on the basis of homology, to be a regulator of sphingolipid synthesis, and in line with this, deletion of this gene perturbed sphingolipid metabolism in the *tms2* mutant. Thus, we reasoned that the deletion might consequently also affect the expression of genes involved in sphingolipid biosynthesis. To determine the expression of genes involved in sphingolipid metabolism, we used genes involved in early steps of sphingolipid synthesis in *Arabidopsis*, which were homologous to those genes in *S. cerevisiae* [29], and then searched for homologs of these genes in rice using *Arabidopsis* and rice database (<http://www.arabidopsis.org/>; <http://www.gramene.org/>). A total of 12 rice genes encoded for four enzymes involved in early steps of sphingolipid synthesis were identified (Table 3) and tested for expression analysis in anthers.

The expression of 9 genes encoding for four enzymes were detected (Fig. 7A–7E), while the primers specific for the other three genes including *serine palmitoyltransferase LOC\_Os01g70370*, *serine palmitoyltransferase LOC\_Os01g70380*, and *ceramide synthase LOC\_Os02g49590* were unable to provide good amplification. Two rice orthologs (*LOC\_Os02g56300* and *LOC\_Os10g11200*) of the *LCB1* subunit of serine palmitoyltransferase (SPT), showed higher expression at the non-permissive (i.e. sterile) temperature (32°C) in the *tms2* mutant compared with the permissive (fertile) temperature (26°C), and these *LCB1* transcripts were higher than those of wild type plants. Another rice ortholog of the *LCB1* (*serine palmitoyltransferase LOC\_Os03g14800*) showed similar expression levels in 26°C and 32°C both in wild type and mutant rice plants, and similar levels of expression in wild type and mutant rice plants (Fig. 7A). Interestingly, a rice ortholog (*serine palmitoyltransferase LOC\_Os11g31640*) of the *LCB2* showed higher expression at permissive (fertile) temperature (26°C) compared with the non-permissive (i.e. sterile) temperature (32°C) in both *tms2* mutant and in the wild type plants, and expressions of this gene were much higher in the mutant than those in the wild type plants at both conditions (Fig. 7B). A rice ortholog (*3-ketosphinganine reductase LOC\_Os02g47350*) of *TSC10*, showed similar levels of expression at permissive (fertile) temperature (26°C) compared with the non-permissive (i.e. sterile) temperature (32°C) in *tms2* mutant, but this gene showed higher expression at permissive (fertile) temperature (26°C) compared with the non-permissive (i.e. sterile) temperature (32°C) in wild type plants (Fig. 7C). A rice ortholog (*sphingolipid-C4-hydroxylase LOC\_Os06g12250*) of *SUR2* showed higher expression at the non-permissive (i.e. sterile) temperature (32°C) compared with the permissive (fertile) temperature (26°C) in the mutant, but in wild type, this gene showed higher expression at permissive (fertile) temperature (26°C) compared with the non-permissive (i.e. sterile) temperature (32°C). Surprisingly, the other tested rice ortholog (*sphingolipid-C4-hydroxylase LOC\_Os02g51150*) of *SUR2* showed higher expression at permissive (fertile) temperature (26°C) compared with the non-permissive (i.e. sterile) temperature (32°C) in both *tms2* mutant and in the wild type plants but expression of this gene were much higher in the mutant than those in the wild type plants at both conditions (Fig. 7D). For *ceramide synthase*, a rice ortholog (*LOC\_Os02g37080*) of *LAC1/LAG1*, showed higher expression at the non-permissive (i.e. sterile) temperature (32°C) in the *tms2*



**Table 2.** Ceramide composition in leaves of *Japonica* WT and RNAi rice lines.

	c16:0	c18:0	c20:0	c20:1	c22:0	c22:1	c24:0	c24:1	c26:0	c26:1
WT	d18:0	0.02±0.00	0.00±0.00	0.02±0.00	0.02±0.00	0.02±0.01	0.20±0.06	0.00±0.00	0.20±0.05	0.01±0.00
	d18:1	0.04±0.00	2.02±1.02	0.02±0.00	1.88±0.15	0.15±0.02	0.98±0.27	0.22±0.02	0.14±0.01	0.07±0.00
	d18:2	0.01±0.01	0.14±0.20	2.13±0.79	55.09±12.86	0.54±0.20	0.17±0.05	5.05±1.16	0.02±0.00	0.61±0.14
	t18:0	0.21±0.07	0.11±0.02	0.50±0.08	0.05±2.86	4.76±1.13	28.87±7.07	0.91±0.09	4.09±1.01	0.22±0.03
	t18:1	0.02±0.00	0.01±0.00	0.05±0.01	0.48±0.53	0.06±0.24	0.36±1.50	1.04±0.22	0.08±0.21	0.11±0.02
3ES-41	d18:0	0.03±0.01	0.01±0.00	0.02±0.00	0.02±0.00	0.01±0.00	0.25±0.03	0.01±0.00	0.21±0.01	0.01±0.01
	d18:1	0.02±0.00	0.01±0.00	0.01±0.00	1.12±0.07	0.09±0.01	0.46±0.05	0.16±0.02	0.05±0.01	0.01±0.00
	d18:2	0.01±0.00	0.10±0.01	2.09±0.26	38.81±4.01	0.55±0.07	0.16±0.02	3.28±0.38	0.01±0.00	0.35±0.07
	t18:0	0.10±0.02	0.06±0.02	0.32±0.02	0.05±0.01	2.26±0.36	12.26±2.07	0.43±0.06	1.64±0.23	0.08±0.01
	t18:1	0.01±0.00	0.01±0.00	0.05±0.00	0.38±0.06	0.04±0.01	0.19±0.04	0.73±0.08	0.04±0.01	0.09±0.01
7ES-4	d18:0	0.03±0.00	0.02±0.01	0.02±0.01	0.03±0.01	0.03±0.00	0.45±0.07	0.02±0.00	0.41±0.11	0.02±0.00
	d18:1	0.04±0.01	0.02±0.00	0.07±0.01	0.69±0.17	0.12±0.04	0.61±0.19	0.24±0.05	0.11±0.04	0.03±0.01
	d18:2	0.03±0.00	0.08±0.01	0.57±0.23	21.46±8.24	0.17±0.07	0.03±0.01	3.31±1.16	0.05±0.01	0.30±0.09
	t18:0	0.25±0.05	0.09±0.05	0.47±0.13	0.18±0.03	3.55±0.75	14.25±4.58	1.12±0.00	2.27±0.00	0.23±0.00
	t18:1	0.04±0.01	0.04±0.01	0.21±0.11	0.37±0.18	0.08±0.04	0.38±0.09	0.99±0.49	0.07±0.02	0.14±0.05
29KS-38	d18:0	0.05±0.02	0.01±0.00	0.03±0.00	0.02±0.00	0.03±0.01	0.60±0.06	0.01±0.00	0.52±0.13	0.07±0.03
	d18:1	0.03±0.01	1.10±1.09	0.04±0.01	1.02±0.20	0.29±0.10	0.45±0.06	0.22±0.09	0.10±0.02	0.02±0.01
	d18:2	0.01±0.00	0.09±0.02	0.92±0.49	14.39±6.71	0.03±0.01	0.02±0.01	3.67±1.35	0.02±0.01	0.42±0.17
	t18:0	0.39±0.08	0.22±0.10	0.94±0.28	0.12±0.02	6.93±2.25	11.22±1.61	0.88±0.13	2.03±0.40	0.16±0.01
	t18:1	0.04±0.01	0.02±0.00	0.03±0.01	0.27±0.04	0.13±0.05	0.54±0.20	0.92±0.42	0.12±0.04	0.08±0.03
29KS-40	d18:0	0.03±0.01	0.00±0.00	0.02±0.00	0.03±0.01	0.04±0.00	0.31±0.01	0.01±0.00	0.27±0.01	0.01±0.00
	d18:1	0.02±0.00	0.73±0.72	0.04±0.01	1.74±0.05	0.12±0.02	0.31±0.05	0.12±0.00	0.05±0.01	0.01±0.00
	d18:2	0.01±0.00	0.14±0.00	0.13±0.00	46.73±0.00	0.03±0.00	0.01±0.00	4.02±0.00	0.01±0.00	0.45±0.00
	t18:0	0.13±0.01	0.10±0.05	0.50±0.02	0.05±0.00	3.02±0.09	10.70±3.06	0.33±0.07	1.25±0.18	0.09±0.02
	t18:1	0.01±0.00	0.02±0.00	0.02±0.01	0.48±0.04	0.03±0.00	0.21±0.01	0.90±0.01	0.05±0.00	0.10±0.00

Unit: nmol g dw<sup>-1</sup>, N: a minimum of 3± SE.  
doi:10.1371/journal.pone.0106386.t002

**Table 3.** Identification of genes involved in early steps of sphingolipid biosynthesis in rice using homologous genes from *A. thaliana* and *S. cerevisiae*.

Gene	<i>S. cerevisiae</i>	<i>A. thaliana</i>	<i>O. sativa</i>
Serine palmitoyltransferase	LCB1	At4g36480	LOC_Os02g56300, LOC_Os10g11200, LOC_Os03g14800
	LCB2	At5g23670	LOC_Os11g31640, LOC_Os01g70380, LOC_Os01g70370
		At3g48780	LOC_Os11g31640, LOC_Os01g70380, LOC_Os01g70370
3-ketosphinganine reductase	TSC10	At3g06060	LOC_Os02g47350
		At5g19200	LOC_Os02g47350
Sphingolipid-C4-hydroxylase	SUR2	At1g14290	LOC_Os06g12250, LOC_Os02g51150
		At1g69640	LOC_Os06g12250, LOC_Os02g51150
Ceramide synthase	LAC1/LAG1	At3g25540	LOC_Os02g37080, LOC_Os02g49590
		At3g19260	LOC_Os03g15750
		At1g13580	-

doi:10.1371/journal.pone.0106386.t003

mutant compared with the permissive (fertile) temperature (26°C), and this expression was much higher than that of wild type plants. In wild type, this gene showed similar levels of expression, with very low level of expression at both conditions. The other tested rice ortholog (*ceramide synthase LOC\_Os02g15750*) of LAC1/LAG1 showed higher expression at permissive (fertile) temperature (26°C) compared with the non-permissive (i.e. sterile) temperature (32°C) in both *tms2* mutant and in the wild type plants, and this gene showed similar levels of expressions in *tms2* mutant and in the wild type plants for each condition (Fig. 7E).

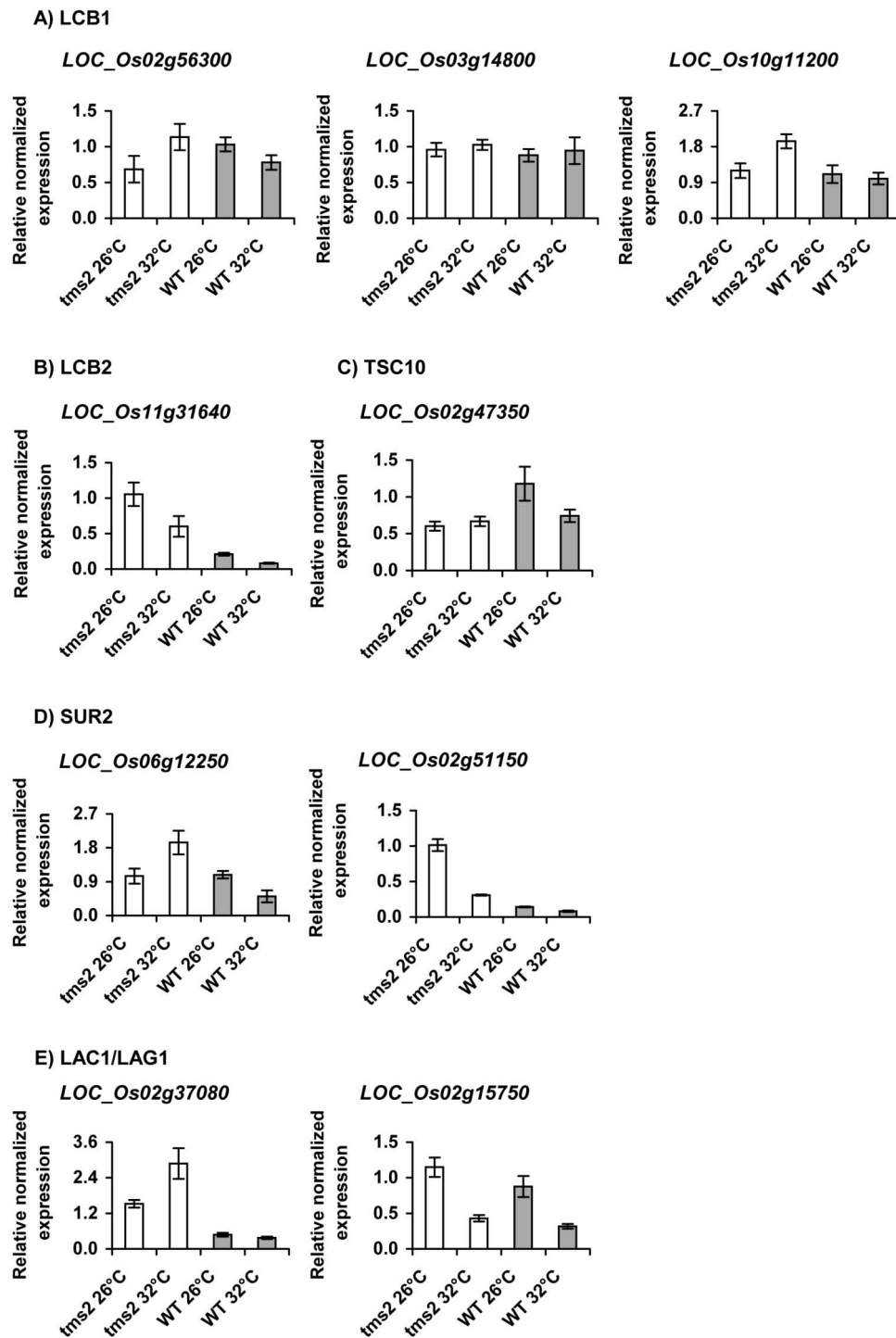
## Discussion

The temperature-sensitive genetic male sterility (TGMS) system provides a powerful tool for the production of hybrid rice, in turn leading to greater yields of this vital crop. However, the molecular mechanism(s) of TGMS are currently unknown, precluding the facile incorporation of this trait into germplasm. A previous study reported a TGMS phenotype of *Ugpl1*-cosuppressed plants resulting from aberrant transcripts, which undergo temperature-sensitive splicing in florets [30]. A rice *ORMDL*, *LOC\_Os07g26940* was predicted to have three splicing variants (<http://www.gramene.org>). Accordingly, we detected all the three transcripts by qRT-PCR. Interestingly, one splice variant showed strongly differential patterns of expression in anther at low and high temperatures, implying a role for *ORMDL LOC\_Os07g26940* in response to temperatures in anther. Importantly, this gene showed high expression in panicle and anthers which, in addition to modulation by temperature, coincides with the TGMS phenotype of *tms2*.

The TGMS phenotype of *tms2* mutant rice plants was reported to be controlled by a recessive gene located in chromosome 7. Previously, the *tms2* mutant plants were reported to contain at least 70 kb deletion including seven genes annotated as expressed proteins [27]. However, only two of these genes (*LOC\_Os07g26930* and *ORMDL LOC\_Os07g26940*) were strongly expressed in panicles and anthers and only the *ORMDL LOC\_Os07g26940* showed differential expression in fertile and sterile conditions in anthers, suggesting a function in this tissue and in TGMS. Accordingly, knock down function of this gene or all *ORMDL* genes including *LOC\_Os07g26940*, *LOC\_Os04g47970*, and *LOC\_Os02g45180*, by RNAi, affected pollen development resulting in male sterility. Reduction of only *ORMDL* gene expression in chromosome 7 (*LOC\_Os07g26940*) or all *ORMDL*

genes affected pollen development in both morphology and staining. Pollen grains of *LOC\_Os07g26940* RNAi and *ORMDL* RNAi were different in sizes, shapes and staining, with none of these plants setting seeds. Interestingly, the male sterility of these RNAi plants was not modulated by temperature, with *LOC\_Os07g26940* RNAi and *ORMDL* RNAi transgenic plants sterile at both low and high temperatures, suggesting that at least one other factor is needed for the conditional male sterility observed in the TGMS phenotype of *tms2*. Thus, deletion of *ORMDL LOC\_Os07g26940* and this (currently unknown) factor are critical for the TGMS phenotype. Although genetic analysis indicates that the TGMS phenotype of *tms2* mutant plants was controlled by a single gene [31,32,27], it is possible that this phenotype is controlled by two closely linked genes not resolved by the current mapping population. Thus, a bigger population with greater recombination maybe required to further study this at the genetic level. Alternatively, the apparent inability of RNAi suppression of *ORMDL* to completely phenocopy the *tms2* TGMS may indicate subtle requirements for the total absence of this gene to deliver conditional fertility.

Recently, *ORMDL* was reported as a negative regulator of sphingolipid synthesis by inhibiting *serine palmitoyltransferase (LCB1/LCB2/TSC3)* [9]. Over expression of *ORM1* and *ORM2* in yeast reduced LCB and ceramide levels. Conversely, deletion of *ORM1/2* elevated the levels of LCB and ceramides [9], although very similar studies have also reported decreased ceramides (in conjunction with elevated LCBs) in yeast mutants lacking *ORMs* [8]. Since a rice *ORMDL* gene is deleted in the *tms2* mutant, an alteration to sphingolipid homeostasis might be predicted. Accordingly, we found that sphingolipid synthesis was perturbed in the *tms2* mutant. Panicles of *tms2* mutant rice plants had higher d18:2, t18:1c, and t18:1t but lower t18:0 than the wild type rice plants, although the overall level of LCBs was not significantly altered. Closer examination of the discrete ceramide species present in the panicles of WT and *tms2* mutant confirmed a concomitant decrease in ceramides levels, in agreement with total ceramide analysis, but also showed some discrete variation in the accumulation of individual ceramides. For example, the predominant C20:1/d18:2 ceramide species is reduced by ~30% in the *tms2* mutant. This is particularly noteworthy, given the total LCB analysis of the *tms2* mutant revealed a moderate increase in this accumulation of this sphingoid base. However, since it is currently unclear if LCB modifying enzymes such as the sphingolipid D8-desaturase and the C4-hydroxylase utilise free LCBs and/or



**Figure 7. Expression analysis of genes involved in sphingolipid synthesis by quantitative RT-PCR.** Expression of genes involved in early steps of sphingolipid synthesis including *LCB1*(A), *LCB2*(B), *TSC10* (C), *SUR2*(D), *LAC1/LAG1*(E) in anthers of *tms2* and wild type rice plants grown in control growth room at 26°C and 32°C. All data are representative from at least two biological repeats, each based on three technical replicates; similar results were obtained in the repeated experiments. Bars indicate the standard error (n=3), which was calculated from technical replicates. Each biological sample was a mixture of 3 plants.  
doi:10.1371/journal.pone.0106386.g007

ceramides as substrates, it is not possible to make causal associations between these two different metabolic pools, or how these are impacted by the *tms2* mutation. It is interesting to note that the rice LAG1/LAC1 ceramide synthase ortholog *LOC\_Os02g37080* showed upregulation in *tms2*, and although the

substrate-specificity of this enzyme remains to be determined, it may play a role in the synthesis of a discrete subset of ceramides as seen in Arabidopsis [16]. More strikingly, and in clear agreement with the yeast studies of Han et al [8], we observed a significant reduction in the ceramide levels in rice plants with perturbation to

the expression of the *ORMDL* gene *LOC\_Os07g26940* – this was true for both the *tms2* mutant and also lines in which RNAi was used to suppress transcript accumulation. On the basis of these two independent interventions, we conclude that *LOC\_Os07g26940* is specifically involved in the sphingolipid homeostasis, most likely in a manner analogous to that proposed by Han et al. [8].

In addition, the deletion in *tms2* mutant increased expression of *LCB1* in high temperature condition compared to that in wild type plants, and increased *LCB2* both in low and high temperature conditions in agreement with its previously established role in yeast [9]. Furthermore, the *tms2* deletion also affected expression of other genes involved in early steps of sphingolipid synthesis such as *SUR2* and *LAC1/LAG1*. Our results indicated that most of the genes involved in early steps of sphingolipid synthesis showed higher expression in the mutant than that in the wild type plants, particularly under high temperature treatment. In Arabidopsis, *LBC1* and *LCB2*, encoding subunits of serine palmitoyltransferase (SPT), were reported as important genes for male gametophyte development, involved in programmed cell death during pollen mitosis [23]. In addition, Arabidopsis *LCB2* null mutants resulted in gametophytic lethality by altering the endomembrane system of pollen and loss of pollen viability, which occurred early in pollen development during transition from the uni-nucleate microspore to the bicellular pollen grain [22]. Deletion of *ORMDL* gene in *tms2* mutant rice plants and RNAi suppression of *ORMDL* gene expression affected pollen development, resulting in sterility. The precise mechanism by which *ORMDL LOC\_Os07g26940* modulates aspects of male fertility still remain to be resolved – based on our data, it appears that, like their yeast orthologs, higher plant ORMs are involved in sphingolipid homeostasis. However, the consequences of release from ORM regulation (reduced ceramides, and an altered LCB profile) is distinct from that observed in yeast, pointing the way for future studies. Given the significantly larger sphingolipidome of plant compared with *S. cerevisiae*, it is perhaps unsurprising that higher plants have evolved additional tiers of regulation for the control of these bioactive lipids.

In conclusion, our results indicate that *tms2* mutant plants lacking one member of the rice *ORMDL* gene family has altered sphingolipid composition. In addition, seven out of the nine expressed genes involved in sphingolipid synthesis pathway showed higher expression in the mutant compared to wild type plants, in agreement with the role of ORM as a homeostatic regulator of sphingolipid biosynthesis [9]. Our results indicate that *tms2* mutant plants lacking *ORMDL LOC\_Os07g26940* affected the expression of several genes involved in early step of sphingolipid synthesis pathway, although the tested genes (orthologous for *LCB1*, *SUR2*, and *LAC1/LAG1*) had discrete expression patterns, suggesting an aspect of sub-functionalization most likely associated with plant-specific sphingolipid homeostasis. Our findings demonstrate an emerging role for *ORMDL* genes in plants as regulators of ceramide accumulation and also provide support for the important role of sphingolipids on pollen development.

## Materials and Methods

### Ethics Statement

N/A

### Plant materials and growth conditions

Wild type rice (*Oryza sativa* ssp *japonica* cv. Nipponbare, and *indica* ssp cv. Pathumthanee1) and *tms2* mutant (in *indica*

background and backcrossed to Pathumthanee1, BC2F4) were planted in natural green house, and about one month before flowering some of these plants (at least 5 plants/lines/condition) were moved to controlled growth rooms at temperature 26°C or 32°C under 80% relative humidity, 12 h light/12 h darkness with a day until flowering.

### Expression analysis by quantitative RT-PCR

For expression of *LOC\_Os07g26930* and *ORMDL LOC\_Os07g26940*, specific primers for *LOC\_Os07g26930* and each transcript of *ORMDL LOC\_Os07g26940* were used for expression analysis in panicle and anther of wild type plants (Pathumthanee 1) grown in 24 and 32°C control growth rooms. Primers specific for *LOC\_Os07g26930* and for each transcript of *ORMDL LOC\_Os07g26940* were designed using Primer-BLAST (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>). In addition, primers specific for each transcript of *ORMDL LOC\_Os07g26940* were used to test expression in other tissues of wild type plants such as root, stem, panicle, leaf, and anther. For expression of all the three *ORMDL* genes in *tms2* and wild type (Pathumthanee 1), primers specific for each *ORMDL* genes, were used and total RNAs were extracted from anther of *tms2* and wild type plants grown in 24 and 32°C control growth rooms. For expression of *ORMDL* genes in RNAi transgenic rice plants, total RNA were isolated from leaf, and qRT-PCR were performed using specific primers for each transcript of *Os07g26940* or primers specific for each of the other two *ORMDL* genes. For expression of genes involved in early steps of sphingolipid synthesis, primers specific for 12 rice genes identified as homologous genes of Arabidopsis, which are homologues to known genes involved in sphingolipid synthesis in *S. cerevisiae* were used for qRT-PCR using total RNAs extracted from anther of *tms2* and wild type plants (Pathumthanee 1) grown in 24 and 32°C control growth rooms. For all the data presented, one sample was a mixture of 3 plants for each genotype. Total RNAs were isolated using TRIZOL™ reagent (Invitrogen) following the manufacture's instructions. First-strand cDNA was synthesized using cDNA synthesis kit (Fermentas) and cDNAs transcribed from the total RNA (20 µl reaction volume). Gene specific primer were designed for amplicons about 200 bp. qRT-PCR experiments were performed on the BIO-RAD CFX 96 real time PCR systems, using SsoFast EvaGreen Supermix (Bio-Rad, Singapore). At the end of each experiment a melting curve was determined for each primer pair at a temperature stage from 60°C to 95°C to check the specificity of annealing. Primers targeting Actin1 (*Os05g36290*) were used to normalize the expression data of each gene in wild type plants. Because *tms2* mutation affects expression of Actin1, thus as recommended [33] primers targeting Elongation Factor 1  $\alpha$ , EF-1 $\alpha$  (*Os03g08020*) were used to normalize the expression data of genes compared expression in the mutant and wild type plants. For each gene, at least two biological replicates were performed, each with three technical replicated. Similar results were obtained in the repeated experiments. The average Ct values and standard deviation were calculated from three technical replicates. The quantification of gene expression was performed using the relative quantification method ( $2^{-\Delta\Delta CT}$ ) [34]. All the tested primers were listed in Table S1.

### Phylogenetic Analysis

Phylogenetic Analysis was performed using protein sequences encoded by the longest splicing forms of each rice *ORMDL* obtained from GRAMENE rice database and *ORMDL* from other species selected from NCBI Reference Sequence database. The tree was generated by neighbour-joining (NJ) algorithm with

p-distance method and pairwise deletion of gap, employing MEGA version 5 [35]. A bootstrap statistic analysis was performed with 1000 replicates to test the phylogeny.

### Generation of *Os07g26940*- RNAi and *ORMDL*-RNAi transgenic rice plants

To generate RNAi construct to suppress expression of *ORMDL* *LOC\_Os07g26940*, all *ORMDL* genes, or *Os07g26930*, a 156-bp fragment specific only for *Os07g26940* cDNA was PCR amplified with primer *Os07g26940*-RNAi-F (5'-ggggtaccactagt CCACT-CACCACGCGCCAC-3', *KpnI*, *SpeI*) and *Os07g26940*-RNAi-R (5'-gggatccgagctc ACGTAGTAGGGGTAGGAC-3', *BamHI*, *SacI*), a 250-bp fragment specific for all *ORMDL* cDNA was PCR amplified with primer *ORMDL*-RNAi-F(5'-ggggtaccactagtGTGAACAAGAACACGGAGT-3', *KpnI*, *SpeI*) and *ORMDL*-RNAi-R (5'-gggatccgagctcTGGTCATCAGCAGCAAAT -3', *BamHI*, *SacI*), and a 422-bp fragment specific only for *Os07g26930* cDNA was PCR amplified with primer *Os07g26930*-RNAi-F(5'-ggggtaccactagtCCAAAAACCGCTCA-CACTCG-3', *KpnI*, *SpeI*), and *Os07g26930*-RNAi-R (5'-gggatccgagctcGAATTCGATCCCAGGTGGCT -3', *BamHI*, *SacI*), which harbors restriction sites (set in lowercase letters) for cloning. The resulting PCR products were first digested by *SpeI* and *SacI* and ligated into vector pTCK303 [28] to get the transitional vector. Then, the PCR products were digested by *KpnI* and *BamHI* and ligated into the transitional vector. The resulting products were sent for sequencing to confirm sense and antisense orientations of the inserts in pTCK303. The constructs were transformed into rice plants (Nipponbare) using an *Agrobacterium*-mediated method.

### Pollen staining and microscopy

Mature pollens were obtained from anthers of 2–3 flowers. Anthers were disrupted on microscope slides using forceps and gently squashed in 4', 6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich) under a coverslip, and visualized with Olympus BX51 microscope.

### Analysis of sphingolipid long-chain bases

LCBs were liberated from 10mg of lyophilized leaves or panicle by alkaline hydrolysis, extraction method [36]. Briefly, this is preformed using 10% BaOH and dioxane 1:1 v/v. The tubes are then capped and placed in a dry block at 110°C for 20 hours. The LCBs are extracted in chloroform/dioxane/water (8/3/8, v/v/v). The LCBs are converted to dinitrophenyl derivatives with 0.2 ml of 0.5% (v/v) methanolic 1-fluoro-2, 4-dinitrobenzene and 0.8 ml of 2 M boric acid/KOH, at 60°C for 30 mins. LCBs are then extracted by phase partitioning with CHCl<sub>3</sub>/methanol/H<sub>2</sub>O, 2:1:1 (v/v/v). The organic phase is removed and washed with an equal volume of 0.1 M KOH and 0.5 M KCl. The organic phase is then blown down and resuspended with methanol, and analyzed by reversed-phase HPLC. Separation was performed using a C18 RP 250×4-mm column with a flow rate of 1 ml/min and a concave gradient from 80 to 100% methanol/acetonitrile/2-propanol, 10:3:1 (v/v/v), against water in 45 min. The elution was monitored with an Agilent 1200 DAD measuring at 350 nm and by ESI-MS/MS MRM on an ABSciex 4000 QTRAP. Three replicate extractions and LCB analysis were performed of each extraction method. Data presented as average (+/– standard error) in ng LCB per mg of dry weight.

### Analysis of ceramide

Samples extracted were ground in liquid N<sub>2</sub> to a fine powder. 10 mg of each sample was used for each replicate analysis. Analysis of the ceramide fraction was performed using LC-MS/MS as previously described [37]. Briefly, the ceramides were extracted in the lower phase of isopropanol/hexane/water (55:20:25 v/v/v) at 60 degrees C. Samples were dried down under a stream of nitrogen. The crude extract was de-esterified in 2 ml of 33% methylamine solution in ethanol/water (7:3 v/v) at 50 degrees for 1 hour. After hydrolysis the samples were dried down under a stream of nitrogen. The ceramides were resuspended in 1 mL of tetrahydrofuran (THF)/methanol/water (2:1:2 v/v/v) and 0.1% formic acid and 50 μL was analyzed on a 4000 QTRAP LC-MS/MS system (ABSciex) after HPLC using an Agilent 1200 fitted with a 100- μL sample loop. Separation was achieved on a SUPELCOSIL ABZ+Plus column 150×3 mm, 5- μm particle size, The sample was eluted at 1 mL min<sup>-1</sup> with a binary gradient system consisting of solvent A, THF/methanol/5 mM ammonium formate (3:2:5v/v/v), and 0.1% formic acid, and solvent B, THF/methanol/5 mM ammonium formate (7:2:1v/v/v), and 0.1% formic acid, held at 40°C. The sample was eluted at 1 mL min<sup>-1</sup> with a binary gradient system consisting of solvent A, THF/methanol/5 mM ammonium formate (3:2:5v/v/v), and 0.1% formic acid, and solvent B, THF/methanol/5 mM ammonium formate (7:2:1v/v/v), and 0.1% formic acid. The gradient started at 40% B rising to 75% B over 10 min. At the end of the gradient, the %B was increased to 100% over 1 min and held at 100% B for an additional 1 min to ensure complete elution of all compounds from the column. The probe was vertically positioned 11 mm from the orifice and charged with 5000 V. The temperature was held at 650°C, GS1 was set at 90 p.s.i., GS2 at 50 p.s.i., curtain gas at 20 p.s.i., and the interface heater was engaged. A minimum of 3 replicate extractions and analysis were performed. Data presented as average (+/– standard deviation). A t-test was performed to determine statistical significance, and in all cases data presented has a p-value of 0.05.

### Supporting Information

**Figure S1** Sequence alignment of the three transcripts of *Os07g26940*. Sequences of rice *ORMDL* were obtained from GRAMENE rice database. (DOC)

**Figure S2** Sequence alignment of the two transcripts of *Os04g47970*. The sequences were obtained from GRAMENE rice database. (DOC)

**Figure S3** Sequence alignment of the representative three transcripts of *ORMDL* genes. Highlight in yellow indicates sequences specific for *Os07g26940*. Highlight in gray indicates sequences used to make RNAi to knockdown all *ORMDL* genes. Green and red indicates primer sequences used for *Os07g26940*-RNAi and *ORMDL* RNAi, respectively. The sequences were obtained from GRAMENE rice database. (DOC)

**Table S1** qRT-PCR primer sequence. (DOC)

### Acknowledgments

We thank Professor Kang Chong from Research Center for Molecular and Developmental Biology; Key Laboratory of Photosynthesis and Environmental Molecular Physiology, Institute of Botany, the Chinese Academy of Sciences, Beijing 100093, China for providing the backbone vector of the

RNAi cassette. We acknowledge Dr. Tharathorn Teerakathiti and Dr. Phanramphoei Namprachan Frantz, for helpful discussion and Dr. Wanarit Wimuttisuk for critical reading of the manuscript and her help with the quantitative RT-PCR. We also thank Dr. Channarong Seepiban and Ms. Amornpan Klanchui for their technical assistances. This work was supported by a grant from Cluster and Program Management Office (CPMO; P-00-20101), National Science and Technology Development Agency (NSTDA).

## References

- Galanter J, Choudhry S, Eng C, Nazario S, Rodríguez-Santana JR, et al. (2008) *ORMDL3* gene is associated with asthma in three ethnically diverse populations. *Am J Respir Crit Care Med* 177: 1194–200.
- Wu H, Romieu I, Sienna-Monge JJ, Li H, del Rio-Navarro BE, et al. (2009) Genetic variation in *ORM1*-like 3 (*ORMDL3*) and gasdermin-like (*GSDML*) and childhood asthma. *Allergy* 64: 629–35.
- Fang Q, Zhao H, Wang A, Gong Y, Liu Q (2011) Association of genetic variants in chromosome 17q21 and adult-onset asthma in a Chinese Han population. *BMC Med Genet* 12(1): 133.
- Moffatt MF, Kabesch M, Liang L, Dixon AL, Strachan D, et al. (2007) Genetic variants regulating *ORMDL3* expression contribute to the risk of childhood asthma. *Nature* 448: 470–473.
- Verlaan DJ, Berlivet S, Humminghake GM, Madore AM, Larivière M, et al. (2009) Allele-specific chromatin remodeling in the *ZBPB2/GSDMB/ORMDL3* locus associated with the risk of asthma and autoimmune disease. *Am J Hum Genet* 85: 377–93.
- Cantero-Recasens G, Fandos C, Rubio-Moscardo F, Valverde MA, Vicente R. (2010) The asthma-associated *ORMDL3* gene product regulates endoplasmic reticulum-mediated calcium signaling and cellular stress. *Hum Mol Genet* 19: 111–121.
- Hjelmqvist L, Tuson M, Marfany G, Herrero E, Balcells S, et al. (2002) *ORMDL* proteins are a conserved new family of endoplasmic reticulum membrane proteins. *Genome Biol* 3: RESEARCH0027
- Han S, Lone MA, Schneider R, Chang A. (2010) *Orm1* and *Orm2* are conserved endoplasmic reticulum membrane proteins regulating lipid homeostasis and protein quality control. *Proc Natl Acad Sci U S A*. 107: 5851–6.
- Breslow DK, Collins SR, Bodenmiller B, Aebersold R, Simons K, et al. (2010) *Orm* family proteins mediate sphingolipid homeostasis. *Nature*. 463: 1048–53.
- Liu M, Huang C, Polu SR, Schneider R, Chang A. (2012) Regulation of sphingolipid synthesis via *Orm1* and *Orm2* in yeast. *J Cell Sci* 125: 2428–2435.
- Spassieva SD, Hille J (2003) Plant sphingolipids today—Are they still enigmatic? *Plant Biol* 5: 125–136.
- Sperling P, Heinz E (2003) Plant sphingolipids: structural diversity, biosynthesis, first genes and functions. *Biochim Biophys Acta* 1632: 1–15.
- Lynch DV, Dunn TM (2004) An introduction to plant sphingolipids and a review of recent advances in understanding their metabolism and function. *New Phytol* 161: 677–702.
- Shi L, Bielawski J, Mu J, Dong H, Teng C, et al. (2007). Involvement of sphingoid bases in mediating reactive oxygen intermediate production and programmed cell death in *Arabidopsis*. *Cell Res* 17: 1030–1040.
- Aubert A, Marion J, Boulogne C, Bourge M, Abreu S, et al. (2011) Sphingolipids involvement in plant endomembrane differentiation: the *BY2* case. *Plant J* 65: 958–71.
- Markham JE, Molino D, Gissot L, Bellec Y, Hématy K, et al. (2011) Sphingolipids containing very-long-chain fatty acids define a secretory pathway for specific polar plasma membrane protein targeting in *Arabidopsis*. *Plant Cell* 23: 2362–78.
- Chen M, Markham JE, Cahoon EB (2012) Sphingolipid  $\Delta 8$  unsaturation is important for glucosylceramide biosynthesis and low-temperature performance in *Arabidopsis*. *Plant J* 69: 769–81.
- Guo L, Mishra G, Markham JE, Li M, Tawfall A, et al. (2012) Connections between sphingosine kinase and phospholipase D in the abscisic acid signaling pathway in *Arabidopsis*. *J Biol Chem* 287: 8286–96.
- Coursol S, Fan LM, Le Stunff H, Spiegel S, Gilroy S, et al. (2003) Sphingolipid signalling in *Arabidopsis* guard cells involves heterotrimeric G proteins. *Nature* 423(6940): 651–4.
- Chen M, Markham JE, Dietrich CR, Jaworski JG, Cahoon EB (2008) Sphingolipid long-chain base hydroxylation is important for growth and regulation of sphingolipid content and composition in *Arabidopsis*. *Plant Cell* 20: 1862–78.
- Chao DY, Gable K, Chen M, Baxter I, Dietrich CR, et al. (2011) Sphingolipids in the root play an important role in regulating the leaf ionome in *Arabidopsis thaliana*. *Plant Cell* 23: 1061–81.
- Dietrich C, Han G, Chen M, Berg HR, Dunn TM, et al. (2008) Loss-of-function mutations and inducible RNAi suppression of *Arabidopsis* *LCB2* genes reveal the critical role of sphingolipids in gametophytic and sporophytic cell viability. *Plant J* 54: 284–298.
- Teng C, Dong H, Shi L, Deng Y, Mu J, et al. (2008) Serine palmitoyltransferase, a key enzyme for de novo synthesis of sphingolipids, is essential for male gametophyte development in *Arabidopsis*. *Plant Physiol* 146: 1322–1332.
- Wang XL, Li XB (2009) The *GhACS1* gene encodes an acyl-CoA synthetase which is essential for normal microsporogenesis in early anther development of cotton. *Plant J* 57: 473–86.
- Imamura Y, Kusano H, Kajigaya Y, Ichikawa M, Shimada H (2007) A rice dihydrosphingosine c4 hydroxylase (*dsh1*) gene, which is abundantly expressed in the stigmas, vascular cells and apical meristem may be involved in fertility. *Plant Cell Physiol* 48: 1108–1120.
- Virmani SS, Kumar I (2004) Development and use of hybrid rice technology to increase rice productivity in the tropics. *International Rice Research Notes* 29: 10–19.
- Pitnjam K, Chakhonkaen S, Toojinda T, Muangprom A (2008) Identification of a deletion in *tms2* and development of gene-based markers for selection. *Planta* 228: 813–822.
- Wang Z, Xu Y, Jiang R, Xu Z, Chong K (2004) A practical vector for efficient knockdown of gene expression in rice (*Oryza sativa* L.). *Plant Molecular Biology Reports* 22: 409–417.
- Zauner S, Termes P, Warnecke D (2010) Biosynthesis of sphingolipids in plants (and some of their functions). *Adv Exp Med Biol* 688: 249–263.
- Chen R, Zhao X, Shao Z, Wei Z, Wang Y, et al. (2007) Rice UDP-glucose pyrophosphorylase1 is essential for pollen callose deposition and its cosuppression results in a new type of thermosensitive genic male sterility. *Plant Cell* 19: 847–61.
- Yamaguchi Y, Ikeda R, Hirasawa H, Minami M, Ujihara A (1997) Linkage analysis of thermosensitive genic male sterility gene, *tms-2* in rice (*Oryza sativa* L.). *Breeding Sci* 47: 371–373.
- Lopez MT, Toojinda T, Vanavichit A, Tragoonrun S (2003) Microsatellite markers flanking the *tms2* gene facilitated tropical TGMS rice line development. *Crop Sci* 43: 2267–2271.
- Caldana C, Scheible WR, Mueller-Roeber B, Ruzicic S (2007) A quantitative RT-PCR platform for high-throughput expression profiling of 2500 rice transcription factors. *Plant Methods* 3(1): 7 doi: 10.1186/1746-4811-3-7.
- Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the  $2^{-\Delta\Delta C_T}$  Method. *Methods* 25: 402–408. doi: 10.1006/meth.2001.1262.
- Tamura K, Peterson D, Peterson N, Stecher G, Nei M, et al. (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* 28: 2731–2739. doi: 10.1093/molbev/msr121.
- Termes P, Sperling P, Albrecht S, Franke S, Cregg JM, et al. (2006) Identification of fungal sphingolipid C9-methyltransferases by phylogenetic profiling. *J Biol Chem* 281 5582–5592.
- Markham JE, Jaworski JG (2007) Rapid measurement of sphingolipids from *Arabidopsis thaliana* by reversed-phase high-performance liquid chromatography coupled to electrospray ionization tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* 21: 1304–1314.

## Author Contributions

Conceived and designed the experiments: AM. Performed the experiments: CC KP KC SC NS KS MS LM JN. Analyzed the data: AM LM JN. Wrote the paper: AM JN.