Small RNAs Originated from Pseudogenes: cis- or trans-Acting?

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Abstract

Pseudogenes are significant components of eukaryotic genomes, and some have acquired novel regulatory roles. To date, no study has characterized rice pseudogenes systematically or addressed their impact on the structure and function of the rice genome. In this genome-wide study, we have identified 11,956 non-transposon-related rice pseudogenes, most of which are from gene duplications. About 12% of the rice protein-coding genes, half of which are in singleton families, have a pseudogene paralog. Interestingly, we found that 145 of these pseudogenes potentially gave rise to antisense small RNAs after examining ~1.5 million small RNAs from developing rice grains. The majority (>50%) of these antisense RNAs are 24-nucleotides long, a feature often seen in plant repeat-associated small interfering RNAs (siRNAs) produced by RNA-dependent RNA polymerase (RDR2) and Dicer-like protein 3 (DCL3), suggesting that some pseudogene-derived siRNAs may be implicated in repressing pseudogene transcription (i.e., cis-acting). Multiple lines of evidence, however, indicate that small RNAs from rice pseudogenes might also function as natural antisense siRNAs either by interacting with the complementary sense RNAs from functional parental genes (38 cases) or by forming double-strand RNAs with transcripts of adjacent paralogous pseudogenes (2 cases) (i.e., trans-acting). Further examinations of five additional small RNA libraries revealed that pseudogene-derived antisense siRNAs could be produced in specific rice developmental stages or physiological growth conditions, suggesting their potentially important roles in normal rice development. In summary, our results show that pseudogenes derived from protein-coding genes are prevalent in the rice genome, and a subset of them are strong candidates for producing small RNAs with novel regulatory roles. Our findings suggest that pseudogenes of exapted functions may be a phenomenon ubiquitous in eukaryotic organisms.

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Introduction

Pseudogenes are genomic sequences derived from functional genes, but are often considered non-functional due to the accumulation of various deleterious mutations over their evolutionary history [1–6]. Compared with its parental gene (more precisely, the direct descendant of the ancestral gene that gave rise to the pseudogene), a pseudogene generally contains sequence features such as premature stop codon or frameshift mutations, due to relaxation of or entirely lack of functional constraints. Two major classes of eukaryotic pseudogenes have been described: processed and duplicated. Processed pseudogenes arose from retrotransposition events, i.e., the insertions of DNA materials into a genome via RNA intermediates. Duplicated pseudogenes, on the other hand, originated from DNA duplications. As a result, duplicated pseudogenes often retain the exon-intron structures of their parent genes, a characteristic absent in processed pseudogenes [1,2,4,6].

Genome-wide pseudogene annotations have been carried out for several mammals and prokaryotes but to date not for rice or any other plants [3,5,7–11]. While previous studies have demonstrated that retrotransposition is the major mechanism for generating mammalian pseudogenes [3,4,6], it remains to be established whether duplication or retrotransposition is the predominant mechanism in plants such as rice.

Rice (Oryza sativa) is a very important crop species that supports about one half of the human population. The genomes of two subspecies (indica and japonica) were sequenced completely in 2005 [12,13]. Based on current annotation (TIGR V5), the rice (japonica) genome contains 41,046 protein coding genes (excluding transposable elements, TE), 763 RNA genes, and 2,859 novel genes seemingly unique to rice and other cereal. To this date, no systematic and comprehensive annotation of pseudogenes has been done to address their impact on the structure and the function of the rice genome. However, a number of rice duplicated pseudogenes have been reported lately, including several arising from MADS-box genes, which encode a large family of transcription factors [7], and 99 pseudogenes in Cyt P450 family [8]. Moreover, current annotation has identified 15,232 TE-related genes and retrotranspositions have been documented to
siRNAs from Rice Pseudogenes

Author Summary

Pseudogenes are “defunct” copies of protein-coding genes that have been accumulated in a genome. They have conventionally been considered the junk byproducts of genome evolution, as they cannot code for proteins due to sequence degeneration. Recent important studies, however, have discovered that a subset of them have unforeseen roles as sources of non-coding RNA transcripts that can regulate the expression of functional coding genes. In this work, we have explored this perspective by studying to what extent rice pseudogenes can encode small RNAs, especially the antisense type. After cross-examining thousands of rice pseudogenes and several millions of small RNA sequences, we found that pseudogene-derived small RNAs are abundant in rice, and furthermore, many of them may function as natural antisense RNAs by interacting complementarily (based on sequence similarity) with sense RNAs from either parental genes or other pseudogenes. Some of these pseudogene RNAs may also be involved in repressing pseudogene transcription. Our findings shed new light on the multifaceted biological roles of pseudogene-derived siRNAs that may or may not affect their functional counterparts directly, indicating that more studies are required to explore in molecular detail the diverse functions of siRNAs originating from pseudogenes in both animals and plants.

Results

Pseudogenes are prevalent in the rice genome

We have assigned a total of 11,956 pseudogenes, most of which contained premature stop codons and frameshift mutations (Table 1), in the rice genome using the PseudoPipe software [48]. Of the 41,046 non-TE-related protein-coding genes used as our query sequences, 4,946 (12.1%) had at least one pseudogene. Respectively, 3,392 and 2,330 of the rice pseudogenes were classified as processed pseudogenes and duplicated pseudogenes by PseudoPipe (Table 1). The rest (6,214) were designated as pseudogene fragments, since they did not contain sequence features that were considered by PseudoPipe to distinguish between retrotranspositions and DNA duplications. Such fragments are usually derived from duplications, so together with duplicated pseudogenes they are referred as non-processed
pseudogenes. The average nucleotide sequence identity between pseudogenes and their parents was 70.5%, 76.6%, and 74.0% for processed pseudogenes, duplicated pseudogenes, and pseudogene fragments, respectively. The mean alignment coverage on the parental genes was 87.9%, 59.6%, and 35.2% for processed, duplicated, and fragments, respectively, suggesting that most past retrotranspositions generated rice processed pseudogenes of full length (in relation to the CDS of their parental genes). Detailed information for individual pseudogenes can be found in the annotation file available on the web (http://www.pseudogene.org/rice09/).

Overall, the processed pseudogenes are randomly distributed in the rice genome, a pattern apparently different from the genome-wide distribution of non-processed pseudogenes (Figure 1 and Supplementary Figure S1). Moreover, non-processed pseudogenes are prevalently located in duplicated regions as expected from well-documented extensive past duplication events in the rice genome. For example, a comparison of our pseudogene annotation with the whole genome duplication (WGD) data, described in [49], showed that 6,071 (70.9%) of rice non-processed pseudogenes are located in WGD regions, versus 2,085 (61.5%) of processed pseudogenes, in consistent with their distinct generation mechanisms ($p = 1.88\times10^{-8}$).

It has been reported that most of the rice genes can not be clustered into gene families [12,13,50], i.e., they do not have a functional rice paralog and thus are considered singleton genes. This is somehow counterintuitive with respect to the past active DNA duplications in rice. To investigate how this has impacted pseudogene biogenesis, we transferred the family annotation of each parental gene to its pseudogene(s), using gene family annotation from TIGR v5. Interestingly, this analysis showed that more than 65% of pseudogenes ($\approx 50\%$ of coding genes) were from singleton gene families (Figure 2), and conversely 13.3% of singleton genes ($\approx 12.1\%$ for all rice genes) had at least one pseudogene. These numbers imply that rice pseudogenes preferentially come from singleton families. Upon more careful investigation, however, we found that this bias was introduced largely by the specific method employed in annotating rice gene families, which clustered genes based on domain architectures of their protein products [50]. As a consequence, two genes sharing all but one domain would have been assigned to distinct families. We therefore utilized an alternative method that is better suited for our purpose in designating singleton genes: a gene is considered singleton if it does not share significant sequence similarity (BLAST e-value $<1\times10^{-14}$) with other rice genes. By this criterion we found that only 7.6% ($1,262$) of singleton genes had at least one pseudogene relative even though they do not have a functional paralog. In conjunction with the TIGR rice gene annotation, our analysis suggests that the overabundance of singletons in rice genome is the outcome of sequence loss rather than sequence degeneration (i.e., pseudogenization).

Overall, we found that family size was negatively correlated with the number of pseudogenes in a family ($R = -0.93, p<1\times10^{-15}$; Spearman’s correlation computed for family size of 2 to 15), suggesting that large gene families do not necessarily have more “dead” (pseudogene) members than small families. However, this negative correlation does not hold for very small gene families (family size 2 to 5; Figure 2), an interesting observation to be further studied.

The top ten genes with the most pseudogenes are LOC_Os01g10030 (550 pseudogenes), LOC_Os09g23670.1 (226), LOC_Os10g14500.1 (125), LOC_Os03g32980.1 (113), LOC_Os11g14500.1 (90), LOC_Os06g11360.1 (90), LOC_Os11g10210.1 (89), LOC_Os11g36210.1 (79), LOC_Os08g39680.1 (73), and

| Table 1. Summary of rice non-RE-related pseudogenes annotated by the program PseudoPipe. |
|---------------------------------------------------------------|
| **Pseudogene Type** | **Number of Pseudogenes** | **Numbers of Parents** | **Pseudogene Length (bp)** | **Sequence Identify (%) between Pseudogenes and Parents** | **Number of Stop Codons in Pseudogenes** | **Number of Frameshifts in Pseudogenes** |
|---------------------------------------------------------------|
| Duplicated | 2350 | 1672 | 748.3 (±619.3) | 76.6 (±614.3) | 12 (±1.9) | 1.2 (±1.9) |
| Processed | 3392 | 1343 | 452.3 (±330.6) | 70.5 (±452.3) | 1.3 (±1.6) | 1.3 (±1.6) |
| Fragment | 6214 | 2580 | 282.2 (±195.4) | 74.0 (±282.2) | 0.9 (±1.3) | 1.0 (±1.4) |

| **Number of Frameshifts in Pseudogenes** | **Pseudogene Coverage (%) on Parents** | **Sequence Identity (%) between Pseudogenes and Parents** | **Number of Stop Codons in Pseudogenes** | **Number of Frameshifts in Pseudogenes** |
|-----------------------------------------|---------------------------------------|-----------------------------------------------|----------------------------------------|----------------------------------------|
| 1.7 (±1.7) | 1.7 (±1.7) | 0.9 (±1.3) | 1.0 (±1.4) | 1.0 (±1.4) |

| **Number of Stop Codons in Pseudogenes** | **Number of Frameshifts in Pseudogenes** |
|-----------------------------------------|----------------------------------------|
| 1.2 (±1.9) | 1.2 (±1.9) |
| 1.3 (±1.6) | 1.3 (±1.6) |
| 0.9 (±1.3) | 1.0 (±1.4) |
The functions for eight of them have not been annotated but two appear to be housekeeping genes. This is strikingly different from what has been reported in mammalian genomes, where a large fraction of pseudogenes are derived from known gene families such as ribosomal protein genes and olfactory receptor genes [3,5]. More specifically, the ribosomal protein genes have generated about 2,000 pseudogenes in both humans and mice [51] but only 50 in rice based on our current study. As the significant enrichment of processed pseudogenes from housekeeping genes in mammals is considered to be relevant to their high expression levels, current finding suggests that distinct evolutionary events are probably responsible for the generation and subsequent retention of pseudogene populations in plants and animals.

**Figure 1.** Genome-wide distribution of rice pseudogenes, siRNAs from developing rice grains, and repeats. Data shown here is for chromosome 1 only, but data for all chromosomes can be found in Supplementary Figure S1 and our website. Moving windows (50,000-nt length and 10,000-nt increment) were employed to calculate the proportion of nucleotides covered by processed pseudogenes (A), non-processed pseudogenes (B), non-TE coding genes (E), annotated TE coding genes or repeats annotated by the RepeatMasker program (F), or the number of siRNAs (C, D) in each window. The black lines at the top of B illustrate whole genome duplication regions and the open ovals mark the centromere. doi:10.1371/journal.pcbi.1000449.g001
Some pseudogenes produce small RNAs in developing rice grains

Recent studies have revealed that pseudogenes are an important source of non-coding RNAs [6,9,26]; furthermore, genome-wide small RNA analyses have indicated that pseudogene-originated siRNAs could either regulate the expression of their parental genes in mice [9,26] or be implicated in silencing pseudogenes themselves in Arabidopsis [41]. To explore this, we have searched a library of small RNAs from developing rice grains [35] for antisense RNAs from pseudogene loci. Such RNAs would be complementary to the mRNAs from parental genes and thus may function as nat-siRNAs. We found that 2,867 and 2,582 pseudogenes had at least one small RNA mapped to their sense and antisense strands, respectively. By a threshold of 4 small RNAs/100 nucleotides (corresponding to 1× coverage, see Materials and Methods for details), we considered 145 pseudogenes as good candidates for producing antisense small RNAs (Figure 3, group A). Three-quarters (75.9%) of these candidates were from singleton families, in consistent with their abundant representation in the rice pseudogene population (Figure 2B). Additionally, 24 (16.6%), 39 (26.9%) and 82 (56.5%) of these pseudogenes were processed, duplicated and fragments, respectively, indicating a slight bias (p = 0.2) of non-processed pseudogenes in generating antisense small RNAs. For the convenience of description, we will use small RNAs and siRNAs interchangeably below even though the functions of the small RNAs in our dataset have not been established experimentally.

Some rice pseudogene siRNAs show an RDR2/DCL3-dependent feature

The sizes of antisense siRNAs, however, suggest that most of the rice pseudogene-derived siRNAs may not serve as nat-siRNAs, as the majority (53.4%) of them are 24-nt long (Figure 4), which is a common signature feature of small RNAs derived from plant RDR2 pathway. Our finding is rather consistent with what has recently been shown for pseudogene-derived siRNAs in Arabidopsis that were predominantly 24-nt long and depended on RDR2 and DCL3 for their accumulation [41]. The potential involvement of RDR2/DCL3 pathway is further supported by the observations that the majority of the 145 pseudogenes also produced some sense small RNAs and that the size distributions of sense and antisense small RNAs were identical (Figure 4). All together, these data indicate that ~50% of pseudogene siRNAs in rice grains could have been produced by the Pol IV/RDR2/Pol V pathway. As plant 24-nt siRNAs and the Pol IV/RDR2/Pol V pathway are specifically implicated in RNA-directed DNA methylation (RdDM) and heterochromatin formation, two processes important for silencing transposons and other retroelements in plants [42–47], these results suggest that some pseudogene siRNAs may be important for cis-repression (more precisely, local-repression) of pseudogene transcription.

Many rice pseudogene siRNAs show features distinct from ra-siRNAs

More careful analysis of small RNA sizes suggested that pseudogene-derived siRNAs might have other regulatory roles besides cis-acting repression in rice. It is known that trans-acting siRNAs (tasiRNAs) or nat-siRNAs in plants exhibit a size range quite different from that of repeat-associated siRNAs. For example, a recent analysis of rice nat-siRNAs found that their lengths varied from 17- to 31-nt with a detectable peak in 21-nt [36]. Interestingly, small RNAs from rice miRNA loci also displayed two peaks, at 21 nt and at 24 nt (Figure 4). In contrast, small RNAs from repeats (LINE, SINE, LTR and DNA transposons) exhibited a single strong peak at 24 nt (Figure 4). Therefore, we decided to study 24-nt and non 24-nt small RNAs separately, with the assumption that these two groups are generated from different pathways and have largely distinct functions. A comparison of the genome-wide distributions of these two groups of small RNAs, to our surprise, did not find an enrichment of 24-nt siRNAs in the centromeric and pericentromeric regions where repetitive elements are concentrated (Figure 1). These data accumulatively suggest that the repeat-relevant RDR2 pathway is not necessarily the sole pathway contributing 24-nt siRNAs to our small RNA library, and that pseudogene-derived siRNAs may have functions other than inducing local heterochromatin formation. It is worth to mention here that the first example of trans-acting nat-siRNAs, formed between SRO5 and P5CDH transcritps through the action of RDR6/DCL2 in Arabidopsis, is indeed 24-nt long [31].

![Figure 2. Percentages of genes/pseudogenes from gene families of different sizes. Genes vs pseudogenes are shown in A and total pseudogenes vs pseudogenes with significant numbers of antisense RNAs (group A in Figure 3) in B. doi:10.1371/journal.pcbi.1000449.g002](image-url)
Complementary small RNAs from rice pseudogenes and their parents

In the studies of siRNAs in mice [9,26], it was considered that simultaneous accumulation of sense-strand small RNAs from parental genes and antisense-strand siRNAs from pseudogenes in the complementary region(s) is good evidence for the production and as well as regulatory potential of pseudogene-derived siRNAs. Following this idea, we have searched for parental gene-pseudogene pairs in which both the parental genes and pseudogenes can produce significant numbers of sense and antisense small RNAs, respectively (see Materials and Methods for details). The complementary interaction of those RNAs can potentially affect the expression of the parental genes (or pseudogenes). Application of this strategy has yielded 38 gene-pseudogene pairs (Figure 3, group B) with the capability of producing trans-nat-siRNAs in developing rice grains. The over representation of non-processed pseudogenes (32 out of 38) lends a good support to our hypothesis, since small RNAs of trans-regulatory function arising from duplicated pseudogenes have previously been documented [6,41,52]. Again, one caveat here is that many of these 38 group B pseudogenes produced both sense and antisense siRNAs with a mixture of 24 and non-24 nt (Table 2). Based on sign-test statistical analysis, we found that 8 of the 38 group B pseudogenes (vs 16 of the 145 group A) had significantly more non-24 than 24 antisense siRNAs (at the false discovery rate of 5%), indicating that a RDR2 independent pathway is very likely involved. As an extra line of evidence for the existence of regulatory pseudogene siRNAs in rice grains, we found that none of the parental genes of the 38 group B pseudogenes had a matching EST from either rice...
| Pseudogene | Parent gene | Chr Start | Locus ID | Sense RNA density | Antisense RNA density |
|-----------|-------------|-----------|----------|-------------------|-----------------------|
| NP1*      | LOC_Os10g04950.1 | chr10     | 2389446  | 0.407692          | 0.853846              |
| NP2*      | LOC_Os01g07180.1 | chr1      | 2579088  | 0.118423          | 0.24898               |
| NP3*      | LOC_Os01g07180.1 | chr1      | 2579088  | 0.118423          | 0.24898               |
| NP4*      | LOC_Os01g07180.1 | chr1      | 2579088  | 0.118423          | 0.24898               |
| NP5*      | LOC_Os01g07180.1 | chr1      | 2579088  | 0.118423          | 0.24898               |
| NP6*      | LOC_Os01g07180.1 | chr1      | 2579088  | 0.118423          | 0.24898               |
| NP7*      | LOC_Os01g07180.1 | chr1      | 2579088  | 0.118423          | 0.24898               |
| NP8*      | LOC_Os01g07180.1 | chr1      | 2579088  | 0.118423          | 0.24898               |
| NP9*      | LOC_Os01g07180.1 | chr1      | 2579088  | 0.118423          | 0.24898               |
| NP10*     | LOC_Os01g07180.1 | chr1      | 2579088  | 0.118423          | 0.24898               |
| NP11*     | LOC_Os01g07180.1 | chr1      | 2579088  | 0.118423          | 0.24898               |
| NP12*     | LOC_Os01g07180.1 | chr1      | 2579088  | 0.118423          | 0.24898               |
| NP13*     | LOC_Os01g07180.1 | chr1      | 2579088  | 0.118423          | 0.24898               |
| NP14*     | LOC_Os01g07180.1 | chr1      | 2579088  | 0.118423          | 0.24898               |
| NP15*     | LOC_Os01g07180.1 | chr1      | 2579088  | 0.118423          | 0.24898               |
| NP16*     | LOC_Os01g07180.1 | chr1      | 2579088  | 0.118423          | 0.24898               |
| NP17*     | LOC_Os01g07180.1 | chr1      | 2579088  | 0.118423          | 0.24898               |
| NP18*     | LOC_Os01g07180.1 | chr1      | 2579088  | 0.118423          | 0.24898               |
| NP19*     | LOC_Os01g07180.1 | chr1      | 2579088  | 0.118423          | 0.24898               |
| NP20*     | LOC_Os01g07180.1 | chr1      | 2579088  | 0.118423          | 0.24898               |
| NP21*     | LOC_Os01g07180.1 | chr1      | 2579088  | 0.118423          | 0.24898               |
| NP22*     | LOC_Os01g07180.1 | chr1      | 2579088  | 0.118423          | 0.24898               |
| NP23*     | LOC_Os01g07180.1 | chr1      | 2579088  | 0.118423          | 0.24898               |
| NP24*     | LOC_Os01g07180.1 | chr1      | 2579088  | 0.118423          | 0.24898               |
| NP25*     | LOC_Os01g07180.1 | chr1      | 2579088  | 0.118423          | 0.24898               |
| NP26*     | LOC_Os01g07180.1 | chr1      | 2579088  | 0.118423          | 0.24898               |
| NP27*     | LOC_Os01g07180.1 | chr1      | 2579088  | 0.118423          | 0.24898               |
| NP28*     | LOC_Os01g07180.1 | chr1      | 2579088  | 0.118423          | 0.24898               |
| NP29*     | LOC_Os01g07180.1 | chr1      | 2579088  | 0.118423          | 0.24898               |
| NP30*     | LOC_Os01g07180.1 | chr1      | 2579088  | 0.118423          | 0.24898               |
| NP31*     | LOC_Os01g07180.1 | chr1      | 2579088  | 0.118423          | 0.24898               |
| NP32*     | LOC_Os01g07180.1 | chr1      | 2579088  | 0.118423          | 0.24898               |
| NP33*     | LOC_Os01g07180.1 | chr1      | 2579088  | 0.118423          | 0.24898               |
| NP34*     | LOC_Os01g07180.1 | chr1      | 2579088  | 0.118423          | 0.24898               |
seeds or seedlings, whereas seven and zero of the 145 group A pseudogenes had ESTs from seeds and seedlings, respectively.

Intriguingly, the pseudogene and its parental gene in 21 of these 38 pairs in group B (55.3%) are very close to each other on chromosomes (<10 kb) (Table 2). By comparison, we found that only 437 of our 11,956 pseudogenes (3.7%) were within 10 kb of their parental genes. These data indicate that siRNAs with a potential of forming (or from) nat-RNA duplex are significantly more likely to be generated from gene-pseudogene pairs of short distances ($\chi^2 = 171.0$, $p < 1.0e-15$). It should be mentioned here that none of these 21 pairs are located in the previously identified siRNA generation hotspots [53]. As an example, one pair defined by the gene LOC_Os10g04950 and its pseudogene 2,594-bp downstream contained large numbers of unique small RNAs exclusively mapped (based on polymorphism) to the sense strand of gene or the antisense strand of pseudogenes (Figure 5, A and B). In this case, we also noticed some antisense RNAs from the parent gene and sense RNAs from the pseudogene. The importance and implication of their presence need to be explored in the future.

Complementary small RNAs from adjacent paralogous pseudogenes

The discovery of nat-siRNAs from gene-pseudogene pairs prompted us to search for other types of mechanisms that could also be important for the production of functional pseudogene siRNAs. It has previously been shown that siRNAs can be derived from hairpin RNAs [9,26,40]. In the search of such small RNAs (Figure 3), we identified two pairs of pseudogenes that potentially could generate transcripts forming hairpin structures (Table 3). One example is a pseudogene located on chromosome 6 (from 12,656,179 to 12,656,385-bp) and its downstream paralogous pseudogene (12,654,917 to 12,654,733-bp; the parent of these two pseudogenes is LOC_Os02g46460). The RNA transcript(s) of these two pseudogenes was predicted to form a 185-nt duplex linked by a 1,283-nt loop (Figure 5). A large number of small RNAs were uniquely mapped to the inverted repeat duplex predominantly to the minus strand, indicating that the transcripts were produced from minus strand of the two pseudogene loci (Figure 5, C and D). We did not detect any small RNAs from the parental gene of these two pseudogenes.

In summary of our analysis of small RNAs from developing rice grains, a total of 145 pseudogenes were identified as good candidates for generating pseudogene-derived antisense endo-siRNAs, with the parental genes for 38 of them harboring significant number of sense siRNAs, suggesting that a small subset of rice pseudogenes might have evolved exapted functions to produce regulatory antisense small RNAs. Our finding extends the previous observations from mouse oocytes [9,26] and Arabidopsis [41], and suggests that siRNA-modulated regulatory function of pseudogenes may be conserved from animals to plants.

Conditional expression of pseudogene-derived siRNAs

We have further explored the diversity of pseudogene-derived siRNAs using additional five small RNA datasets acquired in different rice developmental stages and physiological growth conditions (details in Figure 6 and Supplementary Table S1). Obtained with different high-throughput sequencing techniques, these five datasets contained (a) 285,873 [53], (b) 108,472 [53], (c) 299,454 [54], (d) 182,792 [36], and (e) 11,809 [55] small RNAs, approximately half of which in each dataset could be mapped to the rice genome uniquely (Figure 6). These new datasets are considerably smaller than our primary dataset described above, which had ~1.5 million mapped RNAs, therefore we did not apply the threshold of 4 RNAs/100 nt to them. The numbers of
Figure 5. Examples of regulatory small RNAs derived from pseudogenes. One (A, B) is for gene-pseudogene interaction and the other (C, D) is from a pair of inverted pseudogenes forming predicted RNA duplex. The numbers of small RNAs mapped unambiguously to the top strand (in black) and the bottom strand (in red) are shown in B and D, and the counts are for small RNAs with their starting positions located in a 2-bp bin. Data in the top and bottom of A and B are for the gene (sense) and its pseudogene (antisense), respectively, while the data in the top and bottom of C and D are for a pseudogene and its paralogous pseudogene on the opposite strand. Unique small RNAs from the boxed regions are listed in A and C with yellow columns highlighting unique sites or indels for placing siRNAs specifically to top or bottom sequences.

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pseudogenes with 1–5 antisense small RNAs in these libraries and their comparison with our primary RNA data can be found in the Supplementary Table S1, indicating that 2,350 and 82 rice pseudogenes had ≥1 or ≥5 antisense small RNA in at least one of the five datasets, respectively. Moreover, 43.9% (1,134/2582) of the pseudogenes with ≥1 antisense siRNA(s) in our primary grain library were found to have siRNA(s) in the pool of these five new datasets. Among the 145 group A pseudogenes (Figure 3), 119 (82.1%) could be detected with ≥1 antisense small RNA, and more strictly, 58 (40%) with ≥5 antisense RNAs in at least one of the five datasets (Figure 6A, library all), strongly supporting the existence of endo-siRNAs from those pseudogenes. When considering the source of rice materials used for individual libraries, however, we found that most pseudogenes with antisense siRNAs from rice grains (510 days-after-fertilization) did not have matched siRNAs in other libraries, including those from more developed rice seedling. For example, 91 of the 145 (62.8%) group A pseudogenes were detected with antisense siRNAs from the dehulled grain library obtained by Heisel et al. (Figure 6A, library a), but only 13 of them (9.0%) had siRNAs in the library of 23 days old seedling (Figure 6A, library b). The difference here (p = 1.2e-6) is not simply the consequence of sequencing depth, as library a is not more than three times bigger than library b. Similarly, antisense siRNAs were also detected for most (31) of our 38 group B pseudogenes (Figure 3) in the Heisel’s rice grain dataset (a) but not (only 5) in non grain-related datasets (b, d, e) (Figure 6B). Such skewness is even more pronounced when we examined pseudogenes with more than one siRNA in the new libraries (Figure 6). These comparisons of small RNA libraries from a variety of sources showed that many pseudogenes seemed to produce antisense siRNAs only under specific developmental stages or physiological conditions. Therefore, the pseudogene-derived siRNAs detected only in rice grains may play important roles in early rice grain development, a topic worthy of future investigation.

GO functional categories of rice pseudogene siRNAs.

Our analysis of Gene Ontology (GO) showed that the majority of the parent genes with pseudogenes producing siRNAs in developing rice grains were implicated in gene regulation. For all rice pseudogenes, the most representative function was hydrolase activity (14.8%) (Figure 7A). This bias seems to be specific to plants, but consistent with previous observations for mammalian genomes housekeeping functions such as protein binding (12.4%) and nucleotide binding (9.7%) were also relatively abundant [3,5]. However, it has to be cautioned that these statistics might have been complicated by that fact that 32% of 11,956 pseudogenes have not been assigned a GO functional category. In comparison to all rice pseudogenes, we found that the 145 group A pseudogenes with many antisense siRNAs in developing rice grains were substantially enriched in GO terms implicated in DNA synthesis. We found that 10% of 1,134 pseudogenes with ≥1 antisense small RNAs were involved in DNA replication (12.3%) and DNA modification (11.1%). In contrast, only 1% of 82 pseudogenes with ≥5 antisense small RNAs were involved in DNA synthesis (0.7%). Therefore, pseudogenes with ≥1 antisense small RNAs in rice grains may play important roles in physiological conditions. Therefore, the pseudogene-derived antisense siRNAs only under specific developmental stages or physiological conditions. When considering the source of rice materials used for individual libraries, however, we found that most pseudogenes with antisense siRNAs from rice grains (≤10 days-after-fertilization) did not have matched siRNAs in other libraries, including those from more developed rice seedling. For example, 91 of the 145 (62.8%) group A pseudogenes were detected with antisense siRNAs from the dehulled grain library obtained by Heisel et al. (Figure 6A, library a), but only 13 of them (9.0%) had siRNAs in the library of 23 days old seedling (Figure 6A, library b). The difference here (p = 1.2e-6) is not simply the consequence of sequencing depth, as library a is not more than three times bigger than library b. Similarly, antisense siRNAs were also detected for most (31) of our 38 group B pseudogenes (Figure 3) in the Heisel’s rice grain dataset (a) but not (only 5) in non grain-related datasets (b, d, e) (Figure 6B). Such skewness is even more pronounced when we examined pseudogenes with more than one siRNA in the new libraries (Figure 6). These comparisons of small RNA libraries from a variety of sources showed that many pseudogenes seemed to produce antisense siRNAs only under specific developmental stages or physiological conditions. Therefore, the pseudogene-derived siRNAs detected only in rice grains may play important roles in early rice grain development, a topic worthy of future investigation.

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binding and transcription regulation \( p < 0.05 \) (Figure 7B). This result indicates that pseudogene-derived antisense siRNAs could affect more downstream targets in a broader content by regulating the expression of the parental genes.

**Discussion**

Pseudogenes are conventionally viewed as the by-products of genome evolution. About one-fifth of the rice protein coding genes are transposable elements [14], and the continuous activity of TEs has generated numerous TE-related pseudogenes (~26,000 identified in this study), a feature common to many plant genomes. It is also known that gene duplications have occurred quite frequently in the rice genome [36–38]. For example, it was estimated that 15% to 62% of the rice genome underwent a whole genome duplication (WGD) ~70 million years ago [56,57]. A more recent segmental duplication event has also left ~3 Mb synteny between chromosome 11 and 12 [58,59]. Our observation of a high percentage of non-processed pseudogenes in the rice genome, and particularly their relative enrichment in the WGD region over processed pseudogenes, is consistent with these previous reports. On the other hand, at least 35% of the rice genome is considered the product of retrotransposition events mediated by retrotransposons such as *copia* and *gypsy* elements [13,60]. That estimation is in line with the percentage (28.4%) of processed pseudogenes annotated in current analysis. However, in comparison to the human or rodent genomes, in which about half of their pseudogenes are derived from retrotranspositions, rice has much fewer processed pseudogenes. Moreover, the rice genome has a lower pseudogene to gene ratio (12000 vs 41,000) than the mammalian genomes (approximately 20,000 vs 22,000) [51]. It will be interesting to study in the future how much this is related to the rice genome’s compact size and rapid sequence loss after retrotranspositions or gene duplications [37]. Another avenue to explore is whether many of the predicted rice genes without experimental evidence are actually pseudogenes, an important concern not addressed in current work. Although our pseudogene annotation is largely consistent with the known evolutionary history of the rice genome, the exact ratio of rice processed verse duplicated pseudogenes could deviate from the number reported here, since PseudoPipe [48,61] was originally developed for mammalian genomes.
It is very intriguing that so many rice genes (~50%) are presented themselves as singletons (TIGR v5) [50] even though duplications have been highly active during the evolution of the rice genome. In current work, we found that 13.3% of rice singleton genes have pseudogene relatives despite that the majority (1,729, 64.2%) of them have only one pseudogene. We had expected more singleton genes to have pseudogene relatives, based on high frequency of DNA duplications in rice and the reasonable assumption that most of the duplicated sequences associated with singleton genes should be detected as pseudogenes. The small percentage of singleton genes with pseudogenes (actually 7.6%), based on a more strict definition of singleton family, see Results), in conjunction with the large number of singleton coding genes, indicated that most of the rice sequences resulted from past duplications have been either deleted or altered too substantially to be recognized by simple sequence comparison. This is in full accordance with the general view in the field that deletion and degeneration are the predominant outcome for duplicated sequences [62–64]. Moreover, an analysis of the synonymous substitution ratio (Ks) between pseudogenes and their parental genes showed that the non-processed pseudogenes from singletons (Ks = 0.26±0.39) appear younger than those from multi-gene families (0.46±0.61) (p<0.001), implying that the failure of detecting “old” pseudogenes from singletons might indeed be a reason. As a comparison, we found that 36.5% of Arabidopsis pseudogenes were generated from singleton genes and conversely 11.5% of Arabidopsis singleton genes had a pseudogene relative. These results suggest that domestication process could have introduced significant interference to the nature selection in rice. Nonetheless, there are rice singleton genes that have generated many pseudogenes. For example, the top four singleton genes (LOC_Os01g10030, LOC_Os08g13800, LOC_Os09g02850 and LOC_Os11g25600) with the most pseudogenes have generated a total of 717 pseudogenes, accounting for 6.0% of the rice pseudogenes. This is remarkable especially considering that so many (singleton) genes do not have a protein coding paralog but pseudogene. This is remarkable especially considering that so many rice singleton genes have generated from retrotranspositions, while siRNAs from non-processed pseudogenes can function in trans similar to how some known miRNAs evolving from pseudogenes regulate their targets [6,41,52,65]. Secondly, the same pseudogene might produce both cis-acting (presumably 24-nt) siRNAs and trans-acting (non-24-nt) siRNAs depending on how the pseudogene transcript is processed and utilized by RNAi machinery. Even in the case of cis-acting 24-nt siRNAs, their potential roles in regulating local gene expression should not be ignored. For example, RdDM and formation of repressive chromatin mediated by pseudogene siRNAs can affect the expression of a parental gene if it is sufficiently close to its pseudogene in the chromosomal space due to repression spreading. In our study, we also observed small RNAs derived from adjacent paralogous pseudogenes on opposite strands. Although these pseudogene siRNAs might be taken by the cellular siRNA machinery to modulate the expression of functional genes, they can also be important for suppressing pseudogene expression. It is conceivable that double stranded RNAs can form in vivo between pseudogene antisense siRNAs and their complementary sequences, but only carefully designed molecular and cellular experiments will resolve the different functional scenarios discussed here, for example, by identifying the specific Argonaute proteins and RNA-induced silencing complex (RISC) interacting with pseudogene siRNAs. As most of the studies on 24-nt repeat-associated siRNAs, RDR2/DCL3 and RdDM have been conducted in the model plant organism Arabidopsis, we hope our analysis and similar recent work [35,36] will draw the interests of rice molecular biologists to study endo-siRNAs as there seems to be a difference in the association of 24-nt siRNAs with repetitive regions especially in the centromeric regions between rice and Arabidopsis genomes (Figure 1 here vs Figure 2 in ref 41).

In our current study, we have focused on pseudogenes that produce a significant number of antisense small RNAs. This does not mean that small RNAs from other pseudogene loci are spurious and less likely to be biologically relevant. Rather, the lack of support by multiple RNAs is largely technical, namely the insufficient depth of high-throughput sequencing. For example, 82–92% of the small RNAs from rice grains were only detected once in deep-sequencing using Illumina or 454 sequencing technology [35]. Therefore, low expression and inadequate sampling are probably the reasons for not finding more pseudogene siRNAs. These surely have added a caveat to our results in the comparison of different small RNA datasets with a variety of sequencing depths. On the other hand, pseudogene-derived small RNAs can be much more complicated than what is described here. For example, we found that two pseudogenes with high sequence similarity in very narrow complementary regions could also generate many siRNAs (data not shown). As suggested by previous investigators, such small RNAs may actually be an important source of novel miRNAs [6,11].

Finally, we have also explored the potential regulators for the transcription of these pseudogenes derived endo-siRNAs. In particular, we have examined the genomic distance of pseudogenes to various transposable elements (including DNA transposon, LTR element, LINE and SINE). A total of 1,062 and 1,108 rice pseudogenes were found to be near (~1 kb) active transposable elements (defined as $>$90% of the full length TE) in their 5’ sense and 3’ antisense directions, respectively (Table 4). DNA transposons were the most predominant TEs both in the 5’ sense (78% or 73.3%) and the 3’ antisense directions (81%, 73.2%). The enrichments of these TEs in the flanking regions of pseudogenes are significant based on randomization simulations. However, only seven of the 145 pseudogenes with abundant antisense small RNAs have a TE oriented in the antisense direction in their 3’ flanking regions, suggesting that TE promoters are unlikely the primary
Pseudogene analysis in the rice genome

Materials and Methods

Driver for the production of endo-siRNAs from rice pseudogenes. The initiation of the transcription of these pseudogenes therefore remains perplexing regardless whether the primary transcripts can be subsequently used to produce cis or trans-acting siRNAs.

Materials and Methods

Data source

Rice genomic sequences and their annotations (release 5) were downloaded from the Rice Genome Annotation of TIGR (The Institute of Genomic Research, ftp://ftp.tigr.org). About 5.5 million small RNA sequences, generated by CSIRO (Commonwealth Scientific and Industrial Research Organization) for 1–5 days-after-fertilization (DAF) and 6–10 DAF rice grains using high throughput sequencing [35], were downloaded from the Gene Expression Omnibus (GEO) (GSE11014, http://www.ncbi.nlm.nih.gov/geo/). From this dataset a total of 1,483,951 small RNA sequences matching to rice genome uniquely (i.e., with a single best aligned location in up to one mismatch) were obtained as our primary RNA dataset. Five additional small RNA datasets were also obtained, including (a) 285,873 unique small RNAs from the tissue of rice grains assayed with three replicates of independent libraries and (b) 108,472 small RNAs from rice seedling downloaded from the GEO (GSE13152) [53], (c) 299,454 17-bp MPSS small RNA sequences from University of Delaware (http://mpss.udel.edu/rice/) [54], (d) 182,774 unique small RNAs matching perfectly to the rice genome collected from the library of control (58,781), drought (43,003) and salt (80,990) seedlings [36], and (e) 11,809 small RNA sequences downloaded from CRSDB (Cereal small RNA database, http://sundarlab.ucdavis.edu/smrras/) [55]. Small RNAs from these five additional datasets were mapped to rice genome by the program BLAST [66]. The results were parsed by in-house scripts to extract the chromosomal coordinates of RNAs matching to rice genome uniquely with at most one mismatch in order to ensure comparability between data from our primary library and these additional new libraries. All subsequent analyses utilized genomic coordinates unless specified otherwise.

Pseudogene analysis in the rice genome

For pseudogene assignment, we applied the PseudoPipe program [48,61] to the rice genome with its repetitive sequences masked by the RepeatMasker (http://www.repeatmasker.org). Briefly, PseudoPipe scanned the rice genome for DNA sequences similar to a library of annotated rice protein sequences in the TIGR release V5. After those overlapping with annotated genes were discarded, the rest of matching DNA fragments was assembled into pseudogene candidates based on their structural similarities to the query proteins. Details of the pseudogene discovery procedure have been described previously [48,61]. At the end, a total of 11, 956 pseudogenes derived from non-TE protein coding genes were identified in the rice genome. These pseudogenes were classified into duplicated pseudogenes, processed pseudogenes, and pseudogene fragments based on the potential mechanisms of their generations [3–5]. Thus, each of these pseudogenes was defined by its sequence and structural similarity to a functional gene (or protein), commonly referred to as the parental genes. Our pseudogene annotation is available publicly (http://www.pseudogene.org/rice09/). The sequence identity and coverage (the percentage of parental DNA material present in the alignment) between a pseudogene and its parent are part of the information in our annotations.

Search gene-pseudogene pairs and inverted pseudogene paralogs for potential source of nat-siRNAs

The high sequence similarity between a pseudogene and its parent gene suggests that a natural trans RNA duplex can be formed between the antisense transcript from the pseudogene and the sense transcript from the parent gene. The cellular siRNA/ribozyme machinery can often use such double strand RNAs to produce mature and functional siRNAs. Here we focused our study on antisense small RNAs from pseudogenes by cross-referencing our pseudogene annotation with the library of small RNAs from ≤10 DAF rice grains, which has the largest collection of RNAs among all the publicly available datasets. As described above, we excluded small RNAs that were aligned to more than one location in the rice genome. For each pair of pseudogene and parent gene, their putative coding DNA sequences were first realigned using the global sequence alignment program NEEDLE in the EMBOSS package [67]. We then counted the numbers (N) of small RNAs mapped to the alignment region(s) to generate two numbers for genes, one for the sense and the other for the antisense strand, and likewise two corresponding numbers for

Table 4. Statistics for transposable elements in the flanking region of pseudogenes.

| Pseudogene | S/A | LTR | SINE | LINE | DNA transposon |
|------------|-----|-----|------|------|---------------|
| Duplicated | 5   | 38  | 3    | 1    | 132           |
| A          | 44  | 5   | 0    | 121  |
| Fragment   | 5   | 131 | 14   | 0    | 437           |
| A          | 137 | 16  | 1    | 462  |
| Processed  | 5   | 82  | 13   | 2    | 209           |
| A          | 87  | 5   | 2    | 228  |
| Total      | 5   | 251 | 30   | 3    | 778           |
| A          | 268 | 26  | 3    | 811  |
| Permutation Average | 5   | 107.46 (10.78) | 11.07 (3.29) | 0.76 (0.87) | 387.23 (19.51) |
| A          | 109.02 (9.93) | 11.41 (3.36) | 0.72 (0.82) | 393.98 (19.24) |
| P-value    | 5   | <0.001 | <0.001 | <0.01 | <0.001 |
| A          | <0.001 | <0.001 | <0.01 | <0.001 |

“S” means 5’ sense direction of pseudogene and “A” means 3’ antisense direction. Standard deviation is shown within parentheses.

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pseudogenes. These numbers were then divided by the length of the alignment (L) to obtain the densities (N/L) of small RNAs for each pair of gene and pseudogene. We chose the density of 0.04 as a cutoff in order to select candidate pseudogenes with high potential to produce RNAs. This threshold is equivalent to ~1 × sequence coverage of small RNAs as the length of small RNAs is 18–25 nt [33]. Furthermore, for a pair of parental gene and pseudogene to be considered as a strong candidate with the potential to produce nat-siRNAs, the sense RNAs of the gene and the antisense RNAs of the pseudogene must both meet the density cutoff. The overall protocol and the resulting different groups of pseudogenes are shown in Figure 3.

We also searched for cases in which the transcripts of two adjacent pseudogenes can form RNA hairpin structures. To do so, we used BLASTn program to search paralogs of each pseudogene with antisense RNAs. Paralogous pairs within 2 kb of each other but on opposite strands were evaluated for the potential of producing hairpin RNAs. We used two criteria to define pairs that could generate siRNAs from the RNA hairpin: 1) the stem of the hairpin >100-bp and the loop length <2 kb, and 2) the density of small RNAs (from the same strand) mapped exclusively to the inverted hairpin >0.04 (Figure 3).

Statistical analysis
All statistical analyses were carried out in the R language. When characterizing all pseudogenes, we used the GO function categories of their parents and simply reported GO terms that were highly represented among rice pseudogenes without further inferring statistical significance. To evaluate the functional significance of the 145 pseudogenes enriched with antisense siRNAs (density >0.04 siRNA per nt), we considered the GO distribution of all pseudogenes as the genome-wide background and then employed a re-sampling approach to infer the enrichment of a specific GO term. We selected 145 pseudogenes randomly from all pseudogenes and then recorded the number of pseudogenes in each GO. After 10000 iterations, we obtained an empirical p-value for each GO term measuring the number of iterations that more than X pseudogenes was observed in a GO, where X was the number of pseudogenes in this GO from the 145 pseudogenes.

To identify transposable elements in close proximity of pseudogenes, we considered LTR, SINE, LINE and DNA transposons defined by RepeatMasker annotation with these two criteria: (1) distance to pseudogene is <1 kb and (2) annotated TE covers >90% of its consensus full length (thus considered as ‘active’ TE). So, data discussed here are for pseudogenes located downstream (<1 kb) of an active TE. A re-sampling protocol was used to test the significance of a TE enrichment, which was done by choosing a random region with the same length of a pseudogene from the same chromosome that this pseudogene was located. A total of 11,956 such regions were selected and then the number of TEs within flanking regions of these randomly selected regions was counted. By repeating this process 1,000 times, we derived an empirical p-value for TE enrichments. We carried out this analysis for TEs oriented in the 5‘ sense and 3‘ antisense directions of pseudogenes separately.

Supporting Information
Figure S1 Genome-wide distribution of rice pseudogenes, siRNAs from developing rice grains, and repeats. Found at: doi:10.1371/journal.pcbi.1000449.s001 (10.17 MB DOC)

Table S1 Comparison of pseudogenes with antisense siRNAs in different small RNA libraries. Found at: doi:10.1371/journal.pcbi.1000449.s002 (0.07 MB DOC)

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Author Contributions
Conceived and designed the experiments: XG ZZ MBG DZ. Performed the experiments: XG. Analyzed the data: XG DZ. Wrote the paper: XG ZZ MBG DZ.

References
1. Miglewicz AJ, Smith NR, Robinson PA, Markham AF (2000) Vertebrate pseudogenes. FEBS Lett 468: 109–114.
2. Balakirev ES, Ayala FJ (2003) Pseudogenes: are they “junk” or functional DNA? Annu Rev Genet 37: 123–151.
3. Zhang Z, Harrison PM, Liu Y, Gerstein M (2003) Millions of years of evolution preserved: a comprehensive catalog of the processed pseudogenes in the human genome. Genome Res 13: 2541–2558.
4. Zhang Z, Gerstein M (2004) Large-scale analysis of pseudogenes in the human genome. Curr Opin Genet Dev 14: 329–335.
5. Zheng D, Frankish A, Baertsch R, Kapranov P, Reynold A, et al. (2007) Pseudogenes in the ENCODE regions: consensus annotation, analysis of transcription, and evolution. Genome Res 17: 839–851.
6. Zheng D, Gerstein MB (2007) The ambiguous boundary between genes and pseudogenes: the dead rise up, or do they? Trends Genet 23: 219–224.
7. Nam J, Kim J, Lee S, An G, Ma H, et al. (2004) Type I MADS-box genes have experienced faster birth-and-death evolution than type II MADS-box genes in angiosperms. Proc Natl Acad Sci U S A 101: 1910–1915.
8. Nelson DR, Schuler MA, Paquette SM, Weck- Reichhart D, Bak S (2004) Comparative genomics of rice and Arabidopsis. Analysis of 727 cytochrome P450 genes and pseudogenes from a monocot and a dicot. Plant Physiol 135: 756–772.
9. Tam OH, Aravin AA, Stein P, Giraud A, Murchison EP, et al. (2008) Pseudogene-derived small interfering RNAs regulate gene expression in mouse oocytes. Nature 453: 534–538.
10. Bichler JA, Kast HH (2000) Molecular biology. Slicing and dicing for small RNAs. Science 320: 1023–1024.
11. Sashidharan R, Gerstein M (2008) Genomics: protein fossils live on as RNA. Nature 453: 729–731.

12. Yu J, Wang J, Liu W, Li S, Li H, et al. (2005) The Genomes of Oryza sativa: a history of duplications. PLoS Biol 3: e38.
13. Project IRGS (2005) The map-based sequence of the rice genome. Nature 436: 793–800.
14. Du C, Swigonova Z, Messing J (2006) Retrotranspositions in orthologous regions of closely related grass species. BMC Evol Biol 6: 62.
15. Vanin EF (1985) Processed pseudogenes: characteristics and evolution. Annu Rev Genet 19: 253–272.
16. Yan Y, Saito R, Yoshida N, Yoshiaki A, Wonnswap-Boris A, et al. (2004) A new role for expressed pseudogenes as mRNA regulation of mRNA stability of its homologous coding gene. J Mol Med 82: 414–422.
17. Zheng D, Zhang Z, Harrison PM, Karro J, Carriero N, et al. (2005) Integrated pseudogene annotation for human chromosome 22: evidence for transcription. J Mol Biol 349: 27–45.
18. Harrison PM, Zheng D, Zhang Z, Carriero N, Gerstein M (2005) Transcribed processed pseudogenes in the human genome: an intermediate form of expressed retrosequence lacking protein-coding ability. Nucleic Acids Res 33: 2374–2383.
19. Swenson O, Arvestad L, Lagergren J (2006) Genome-wide survey for biologically functional pseudogenes. PLoS Comput Biol 2: e46.
20. Frith MC, Wilming LG, Forrest A, Kawaji H, Tan SL, et al. (2006) Pseudo-messenger RNA: phantoms of the transcriptome. PLoS Genet 2: e25.
21. Ota T, Neri M (1995) Evolution of immunoglobulin VH pseudogenes in chickens. Mol Biol Evol 12: 94–102.
22. Korosec SA, Park JH, O’Shea M (1999) Neuronal expression of neuronal nitric oxide synthase (nNOS) protein is suppressed by an antisense RNA transcribed from an NOS pseudogene. J Neurosci 19: 7711–7720.
23. Korosec S, O’Shea M (2002) Evolution of nitric oxide synthase regulatory genes by DNA inversion. Molecular Biology and Evolution 19: 1226–1233.
24. Korneev SA, Strzaub V, Kemenes I, Korneeva EI, Ott SR, et al. (2003) Timed and targeted differential regulation of nitric oxide synthase (NOS) and anti-NOS genes by reward conditioning leading to long-term memory formation. Journal of Neuroscience 23: 1188–1192.

25. Duret L, Chureau C, Samain S, Weissenbach J, Avner P (2006) The Xist RNA gene evolved in eutherians by pseudogenization of a protein-coding gene. Science 312: 1653–1655.

26. Watanabe T, Totsuki Y, Toyoda A, Kaneda M, Karamochi-Miyagawa S, et al. (2008) Endogenous siRNAs from naturally formed siRNAs regulate transcripts in mouse oocytes. Nature 453: 539–543.

27. Bartel DP (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116: 281–297.

28. Zamore PD, Haley B (2005) Ribonome: the big world of small RNAs. Science 309: 1519–1524.

29. Pergaite A, Yoshihata M, Wu G, Albrecht HL, Poehing RS (2004) SGS3 and SGS2/DEDE1/RDR6 are required for juvenile development and the production of trans-acting siRNAs in Arabidopsis. Genes Dev 18: 2568–2579.

30. Vaucheret H, Rajagopal R, Lepers C, Gasciolli V, et al. (2004) Endogenous trans-acting siRNAs regulate the accumulation of Arabidopsis mRNAs. Mol Cell 16: 69–79.

31. Borsani O, Zhu J, Vershy E, Sunkar R, Zhu JK (2005) Endogenous siRNAs derived from a natural cis-anti-sense transcripts regulate salt tolerance in Arabidopsis. Cell 125: 1279–1291.

32. Vaquero S (2006) Arabidopsis endogenous small RNAs: highways and byways. Trends Plant Sci 11: 466–468.

33. Buhler M, Haas W, Gygi SP, Moazed D (2007) RNAi-dependent and -independent RNA turnover mechanisms contribute to heterochromatic gene silencing. Cell 129: 707–721.

34. Katiyar-Agarwal S, Gao S, Vivian-Smith A, Jin H (2007) A novel class of endogenous trans-acting siRNAs from a pair of natural cis-antisense transcripts regulate salt tolerance in Arabidopsis. Cell 123: 1279–1291.

35. Zhu QH, Spriggs A, Matthew L, Fan L, Kennedy G, et al. (2008) A diverse set of endogenous trans-acting siRNAs from naturally formed dsRNAs regulate transcripts in Oryza sativa. Genome Res 19: 70–78.

36. Zhou X, Sunkar R, Jin H, Zhu JK, Zhang W (2009) Genome-wide identification and analysis of small RNAs originated from natural antisense transcripts in Oryza sativa. Genome Res 19: 70–78.

37. Aravin AA, Hanon GJ, Brenner EC (2007) The Psoi-pRNA pathway provides an adaptive defense in the transposon arms race. Science 318: 761–764.

38. Houben S, Kammenga LM, Bereznikov E, Cronenbold D, Girard A, et al. (2007) A role for Psoi and pRNAs in germ cell maintenance and transposon silencing in Zea mays. Cell 129: 69–82.

39. Kawamura Y, Saito K, Kim T, Ono Y, Arai K, et al. (2005) Drosohila endogenous small RNAs bind to Argonaut 2 in somatic cells. Nature 453: 793–797.

40. Okamuro K, Chung WJ, Ruby JG, Guo H, Bartel DP, et al. (2008) The Drosohila hairpin RNA pathway generates endogenous short interfering RNAs. Nature 453: 803–808.

41. Kasschau KD, Fahlgen N, Chapman EF, Sullivan CM, Cumbie JS, et al. (2007) Genome-wide profiling and analysis of Arabidopsis siRNAs. PLoS Biol 5: e57.

42. Matzke M, Kann T, Huettel B, Daxinger L, Matzke AJ (2007) Targets of RNA-directed DNA methylation. Curr Opin Plant Biol 10: 512–519.

43. Cho SH, Adams-Quaye C, Corral G, Arai MA, Ma Z, et al. (2008) Phycocyanin gene patens DCL3 is required for 22–24 nt siRNA accumulation, suppression of retrotransposon-derived transcripts, and normal development. PLoS Genet 4: e1000314.

44. Baltimore D (2004) RNA silencing in plants. Nature 431: 356–363.

45. Vaucheret H (2006) Post-transcriptional small RNA pathways in plants: mechanisms and regulations. Genes Dev 20: 759–771.

46. Brodersen P, Voinnet O (2006) The diversity of RNA silencing pathways in plants. Trends Genet 22: 296–303.

47. Chan SW, Henderson IR, Jacobsen SE (2005) Gardening the genome: DNA methylation in Arabidopsis thaliana. Nat Rev Genet 6: 351–360.

48. Zhang Z, Carreno N, Zheng D, Karrer J, Harrison PM, et al. (2006) PseudoPipe: an automated pseudogene identification pipeline. Bioinformatics 22: 1437–1439.

49. Lin H, Zhu W, Silva JC, Gu X, Buell CR (2006) Intron gain and loss in segmentally duplicated genes in rice. Genome Biol 7: R41.

50. Lin H, Ouyang S, Egan A, Nobuta K, Haas BJ, et al. (2008) Characterization of paralogous protein families in rice. BMC Plant Biol 8: 18.

51. Karro JE, Van Y, Zheng D, Zhang Z, Carreno N, et al. (2007) Pseudogene.org: a comprehensive database and comparison platform for pseudogene annotation. Nucleic Acids Res 35: D53–60.

52. Allen E, Xie Z, Gustafson AM, Sungh GH, Spatafora JW, et al. (2004) Evolution of microRNA genes by inverted duplication of target gene sequences in Arabidopsis thaliana. Nat Genet 36: 1282–1290.

53. Hezel SE, Zhang Y, Allen E, Guo L, Reynolds TL, et al. (2008) Characterization of unique small RNA populations from rice grain. PLoS ONE 3: e2871.

54. Nobuta K, Venu RC, Lu C, Belo A, Vemarahju K, et al. (2007) An expression atlas of rice mRNAs and small RNAs. Nat Biotechnol 23: 473–477.

55. Johnson C, Bowman L, Adai AT, Vance V, Sundaresan V (2007) CSRDB: a small RNA integrated database and browser resource for cereals. Nucleic Acids Res 35: D829–833.

56. Paterson AH, Bowers JE, Chapman BA (2004) Ancient polyploidization predating divergence of the cereals, and its consequences for comparative genomics. Proc Natl Acad Sci U S A 101: 9903–9908.

57. Wang X, Shi X, Hao B, Ge S, Luo J (2005) Duplication and DNA segmental loss in the rice genome: implications for diploidization. New Phytol 165: 937–946.

58. Consortia: TRCA (2005) The sequence of rice chromosomes 11 and 12, rich in segmentally duplicated genes. BMC Genomics 3: 20.

59. Guo X, Wang Y, Knightley PD, Fan L (2007) Patterns of selective constraints in noncoding DNA of rice. BMC Evol Biol 7: 208.

60. Vicent CM, Jaakelainen MJ, Kalendari R, Schultmann AH (2001) Active paralogous protein families in rice. Journal of Neuroscience 25: 1188–1192.

61. Zheng D, Gerstein MB (2006) A computational approach for identifying pseudogenes in the ENCODE regions. Genome Biol 7 Suppl 1: S13.1–10.

62. Lynch M, Conery JS (2000) The evolutionary fate and consequences of duplicate genes. Science 290: 1151–1155.

63. Lynch M, Force A (2000) The probability of duplicate gene preservation by subfunctionalization. Genetics 154: 459–473.

64. Wagner A (2001) Birth and death of duplicated genes in completely sequenced eukaryotes. Trends Genet 17: 237–239.

65. Koch K, Blank C, Møller-Skjold P, Møller JH (2005) An automated pseudogene identification pipeline. Bioinformatics 21: 1188–1192.

66. Zwack H, Althaus SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215: 403–410.

67. Rice P, Longden I, Bleasby A (2000) EMBOSS: the European Molecular Biology Open Software Suite. Trends Genet 16: 276–277.