Retrogenes in Rice (*Oryza sativa* L. ssp. *japonica*) Exhibit Correlated Expression with Their Source Genes

Hiroaki Sakai[1,2], Hiroshi Mizuno[1], Yoshihiro Kawahara[1], Hironobu Wakimoto[1,3], Hiroshi Ikawa[4], Hiroyuki Kawahigashi[1], Hiroyuki Kanamori[1], Takashi Matsumoto[1], Takeshi Itoh[1], and Brandon S. Gaut[2,*]

[1]Agrogenomics Research Center, National Institute of Agrobiological Sciences, Tsukuba, Ibaraki, Japan

[2]Department of Ecology and Evolutionary Biology, University of California, Irvine

[3]Hitachi Government & Public Corporation System Engineering, Ltd., Koto-ku, Tokyo, Japan

[4]Tsukuba Division, Mitsubishi Space Software Co., Ltd., Tsukuba, Ibaraki, Japan

*Corresponding author: E-mail: bgaut@uci.edu.

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Abstract

Gene duplication occurs by either DNA- or RNA-based processes; the latter duplicates single genes via retroposition of messenger RNA. The expression of a retroposed gene copy (retrocopy) is expected to be uncorrelated with its source gene because upstream promoter regions are usually not part of the retroposition process. In contrast, DNA-based duplication often encompasses both the coding and the intergenic (promoter) regions; hence, expression is often correlated, at least initially, between DNA-based duplicates. In this study, we identified 150 retrocopies in rice (*Oryza sativa* L. ssp *japonica*), most of which represent ancient retroposition events. We measured their expression from high-throughput RNA sequencing (RNAseq) data generated from seven tissues. At least 66% of the retrocopies were expressed but at lower levels than their source genes. However, the tissue specificity of retrogenes was similar to their source genes, and expression between retrocopies and source genes was correlated across tissues. The level of correlation was similar between RNA- and DNA-based duplicates, and they decreased over time at statistically indistinguishable rates. We extended these observations to previously identified retrocopies in *Arabidopsis thaliana*, suggesting they may be general features of the process of retention of plant retrogenes.

Key words: retroposition, gene expression, gene duplication.

Introduction

Gene duplication is a source of evolutionary novelty (Ohno 1970) and occurs through either DNA- or RNA-based processes. DNA-based processes may lead to the duplication of entire genomes (polyploidy), large chromosomal segments (Prince and Pickett 2002) or, occasionally, individual genes. In contrast, RNA-based processes typically result in the duplication of a single gene. These genes are duplicated by the reverse transcription of an RNA intermediate, followed by integration into DNA.

RNA-based duplication produces new gene copies or "retrocopies." Retrocopies have traditionally been considered nonfunctional ("dead on arrival") because they often lack the regulatory elements of their source (or "parental") genes and contain functional disruptions, such as 5′ truncations, frameshift mutations, and in-frame terminations (Vanin 1985). Analyses of the human genome have revealed that RNA-based duplication is common, with over 3,590 retrocopies in total, but 84% of these retrocopies contain functional disruptions (Vinckenbosch et al. 2006). Nonetheless, a subset of retrocopies is expressed and may have evolved unique functions, particularly in the male germ line (Betran et al. 2002; Emerson et al. 2004; Potrzebowski et al. 2008). Retrocopies also contribute to novel functions within chimeric genes, such as the *jingwei* gene of Drosophila (Long and Langley 1993). Thus, while the majority of retroposition events result in processed pseudogenes, retroposition may play a significant role in the evolution of novelty.

Expressed retrocopies (or "retrogenes") require regulatory elements for transcription. It is thought that retrogenes acquire regulatory functions by one of three mechanisms (Kaessmann et al. 2009). The first is the use, or even cooption, of the regulatory elements of nearby genes. For example, some retrogenes have inserted into introns and are expressed as a fusion transcript under the control of the regulatory elements of the native gene (Long and Langley 1993; Wang et al. 2002). The second mechanism is the acquisition of de novo regulatory elements. One potential source of de novo elements is CpG islands, which sometimes have the capacity to promote transcription (Yamashita et al. 2005; Okamura and Nakai 2008). Finally, retrogenes may inherit promoters and enhancer elements from their parental genes. This is most likely to occur if there is leaky transcription from a start site far upstream from the parental gene, so that retroposition includes 5′ regulatory sequences along with coding regions, resulting in a new gene with regulatory properties similar to the parental gene. Although parental genes have not typically been considered a source for regulatory elements, a recent study has suggested that the retroposition of parental regulatory sequences does occur (Okamura and Nakai 2008).

These three possible modes of promoter acquisition predict different patterns of expression divergence between a retrogene and its parental gene. If, for example, the retrogene has acquired new regulatory elements from nearby genes or from de novo sequences, then the expression patterns of the retrogene and its parental gene should be uncorrelated. Conversely, the retrogene and the parental gene are expected to have correlated patterns of gene expression if the retrogene inherits regulatory regions. Although some studies have documented tissue-specific expression of retrogenes (Marques et al. 2005; Shiao et al. 2007; Rosso et al. 2008a, 2008b), few studies have compared expression between retrogenes and their putative parental genes, particularly on a genome-wide scale.

In this study, we examine the expression of retrogenes in the genome of rice, *Oryza sativa* L. ssp. *japonica*. We have chosen to study rice for three reasons. First, a previous study has documented that retrocopies are abundant in rice (Wang et al. 2006). Second, retrocopies tend to be expressed more often in plants than in animals. Over 80% of retrocopies in rice and poplar are either expressed or inferred to be functional by structural and evolutionary characteristics (Wang et al. 2006; Zhu et al. 2009) compared with only 30% in the human genome (Vinckenbosch et al. 2006). Finally, rice has abundant genomic resources, including genomic sequences of both ssp. *japonica* and ssp. *indica* (International Rice Genome Sequencing Project 2005; Yu et al. 2005) and annotation resources (Tanaka et al. 2008).

We first identify retrocopies in the rice genome through an extensive genomic survey, using conservative approaches to maximize the probability that our designation of retrogenes and their parental genes are accurate. We then examine gene expression in seven rice tissues—leaf, root, shoot, panicle before and after flowering, seed, and callus—by generating high-throughput sequences of mRNA (mRNA-seq). Unlike microarray-based approaches, mRNAseq does not rely on the predefined gene annotations, allowing us to accurately assess expression of unannotated retrocopies. Finally, we utilize mRNAseq data both to compare gene expression patterns between retrogenes and their parents and to compare patterns of expression divergence between RNA-based duplicates and DNA-based duplicates. Overall, we find that expression patterns are surprisingly well conserved between retrogenes and their parental genes, but the molecular and evolutionary mechanisms underlying this correlation are not yet clear.

Materials and Methods

Retrogene Detection

We obtained 40,353 protein sequences of *O. sativa* ssp. *japonica* cv. Nipponbare as well as 9,966 ab initio predicted genes from the Rice Annotation Project Database (RAP-DB) (Tanaka et al. 2008). If there were two or more identical protein sequences in a locus, we selected one sequence with the longest transcript. We removed sequences related to transposable elements (TEs) by two methods. First, given the repeat-masked genome sequence (IRGSP build 5) (http://rapdb.dna.affrc.go.jp/), we discarded a sequence if over 40% of the sequence was masked. Second, we conducted BlastN searches (Altschul et al. 1997) with "-e 1.0e-10" option against RetrOryza sequences (Chaparro et al. 2007); we discarded genes if over 40% of the sequences were covered by any RetrOryza sequence. After this process, we were left with 46,235 nonredundant and non-TE–related protein sequences. We removed TE-related sequences from consideration because we did not want to base our inferences on TEs misannotated as genes or gene fragments captured by TEs.

These 46,235 genes were culled to make an initial set of potential parental genes, based on two criteria: 1) the CDS started with methionine, ended with a stop codon, and spanned 100 or more codons and 2) the transcribed region contained one or more introns of $\geq$70 bp in length with consensus GT-AG splicing motifs. If a gene had only one intron, we selected the sequence as a potential parental gene only if it had no poly-A tract within 500 bp downstream, defining the poly-A tract as a 20 bp window containing 16 or more adenines. After this culling procedure, we identified 19,235 potential parental genes.

To find retrocopies, we mapped the protein sequences of the 19,235 potential parental genes to the rice genome, using TFASTY with default parameters (Pearson et al. 1997). We retained only homologous hits that showed $\geq$35%

identity, covered ≥70% of the parental genes, and lost one or more introns. If the parental gene had only one intron, we retained the hit only if the intron was lost and if there was a poly-A tract within 500 bp downstream. An intron was deemed to be missing if 10 bp upstream and downstream sequences of the exon/intron boundary were successfully aligned without any gaps. We also discarded hits that contained insertions of ≥70 bp relative to the CDS of a parental gene. If hits overlapped on the genome, we selected the hit with the highest identity and sequence coverage.

We applied three additional filters to our set of putative retrocopies. First, we recognized that these retrocopies could be the product of the DNA-based duplication of an intronless gene. To eliminate this possibility, we conducted all-against-all FASTA search, querying the 46,235 nonredundant protein sequences with putative retrocopies. We retained only the homologous hits that showed the highest identity and sequence coverage to the set of potential parental genes. Second, we aligned the remaining retrocopies to their putative parental genes and estimated d_S and d_N with the modified Nei–Gojobori method (Zhang et al. 1998). We discarded retroparent pairs with d_S values (>2.0). For individual gene pairs, the deviation of the d_N/d_S ratio from 1.0 was measured in HYPHY (Pond et al. 2005), by estimating the likelihood of a constrained ($d_N/d_S = 1.0$) and a free model. The HYPHY analyses were based on the HKY85 model of nucleotide substitution (Hasegawa et al. 1985). Finally, we discarded retrocopies that were tandemly located with parental genes, based on the criteria of Hanada et al. (2008).

Identifying DNA-Based Duplicates

For comparison's sake, we identified pairs of duplicate genes. To identify DNA-based duplicates for comparison, we used a procedure similar to that described in Makova and Li (2003). First, all nonredundant protein sequences were subjected to BlastP with default settings. We retained pairs of sequences if 1) the alignable region between them was >80% of the longer protein and 2) the identity (I) between them was $I \geq 30\%$ when the alignable region was longer than 150 aa and I was $\geq 0.01n + 4.8L^{0.32[1 + \exp(-L/1000)]}$ (Rost 1999) for all other protein pairs, where $n = 6$ and L is the alignable length between the two proteins. Based on these pairings, gene families were generated by the Markov Cluster Algorithm (http://micans.org/mcl/).

For each gene family, we calculated d_S for all pairwise combinations and selected the pair with the smallest d_S. We then proceeded by selecting independent pairs (with no overlap with pairs that had already been selected) with increasing d_S. Any duplicate gene pairs with $d_S > 2$ were excluded. We also discarded duplicate gene pairs with $d_S < 0.05$ because it was difficult to estimate expression level accurately when sequence identity between two gene copies was too high. Finally, we discarded tandemly located duplicate genes (Hanada et al. 2008).

Expression Analysis

We performed mRNAseq on seven tissues of *O. sativa* ssp. *japonica* cv. Nipponbare: callus, leaf, root, panicle before flowering, panicle after flowering, seed, and shoot. We obtained seeds (accession number "JP229579") from the GENE bank in the National Institute of Agrobiological Sciences, Japan (http://www.gene.affrc.go.jp/about_en.php). The seeds were germinated in a growth chamber at 28 °C under a 16-h light/8-h dark regime. Seven days after germination, shoots and roots were collected. In the meantime, plants were grown in paddy fields, where leaves (7 days before heading to 7 days after flowering) and panicles (7 days before and 0–7 days after heading to flowering) were collected. Callus was induced on N6D medium according to a previous protocol (Ozawa 2009). For RNA extraction from each plant tissue, at least 10 plants were collected, immediately frozen in liquid nitrogen, and mixed, to minimize the effect of transcriptome unevenness among plants. Total RNA was extracted from each tissue with the RNeasy Plant mini kit (Qiagen), and cDNA was synthesized with mRNAseq 8-sample prep kit (Illumina) (Mizuno et al. 2010). We constructed cDNA libraries for the seven tissues individually and sequenced single ends of each on the Illumina GAIIx platform for four separate runs (three at 36 cycles and one at 76 cycles). The sequence data have been submitted to the DNA Data Bank of Japan (DDBJ) Sequence Read Archive (DRA) (http://trace.ddbj.nig.ac.jp/dra/index_e.shtml) under accession numbers DRR001024–DRR001051.

After sequencing, we discarded 3′-end nucleotides of low quality reads so that every read was at least 35 bp in length and had three or more successive nucleotides with the quality score ≥ 20 at the 3′-end. We also trimmed sequencing adapters. Quality and adapter trimming were performed by customized C and Perl programs. Trimmed reads were mapped to the genome sequence with the bwa program (Li and Durbin 2009) using default settings. Only uniquely mapped reads with ≤2 mismatches were retained for further analyses. For each retrocopy, parental gene and DNA-based duplicate gene, we calculated the number of reads per kilobase (RPK) with a customized Perl program.

In order to determine the background level of expression, we investigated the number of mRNAseq reads mapping to intergenic regions. To do this, we first selected intergenic regions of ≥3 kb in length and extracted 1 kb from the midpoint of the regions. To ensure these were not protein coding regions, we conducted BlastX search against the nonredundant protein database (nr) of the National Center for Biotechnology Information, with a cutoff E value of 10^{-10} and discarded sequences with hits. For the remaining 1,885 1-kb intergenic sequences, we calculated the number of overlapping mRNAseq reads. The read counts were then normalized using the edgeR package (Robinson et al. 2010). Based on these analyses, we determined that the mean RPK value per tissue in intergenic regions was 10; this RPK value defined the background level.

Tissue Specificity

In order to evaluate the tissue specificity of each gene, we calculated Shannon entropy based on RPK values (Kadota et al. 2006). We estimated one-step Tukey biweight *tbw* (Mosteller and Tukey 1977; Sachs 1994) for each gene, which was defined as

$$tbw(X_1, X_2, \ldots, X_N) = \frac{\sum_{i=1}^{N} W_i \times X_i}{\sum_{i=1}^{N} W_i},$$

where $N = 7$ is the number of tissues and X_i is the RPK value for the *i*th tissue. The weight, W_i, was given by

$$W_i = (1 - Z_i^2)^2 \quad if|Z_i|<1, \; 0\,\text{otherwise},$$

where Z_i is given by

$$Z_i = \frac{X_i - m}{5 \times \text{MAD} + 0.0001},$$

where *m* is a median of the RPK values and MAD is a median absolute deviation. We applied 5 as a multiplicative factor and added 0.0001 to avoid division by zero. Before calculating *tbw*, we assigned a randomly chosen value of $<10^{-5}$ to the tissues with zero RPK values to avoid zero median. We identified tissue-specific expression for each gene using Ueda's Akaike's Information Criterion–based method (Kadota et al. 2003).

Arabidopsis thaliana Retrogene Analyses

We obtained 63 pairs of retrocopies and parental genes of *A. thaliana* from Zhu et al. (2009). Protein sequences of the retrogenes and parental genes were aligned by ClustalW2 (Chenna et al. 2003), and codon alignments were constructed by PAL2NAL (Suyama et al. 2006). We estimated d_S (Zhang et al. 1998) and discarded pairs with $d_S > 2.0$, resulting in 48 pairs. Protein and nucleotide sequences were retrieved from TAIR (http://www.arabidopsis.org/). For expression analyses, we obtained microarray expression data from 55 samples (Schmid et al. 2005; Matsuda et al. 2010). We calculated the Pearson product-moment correlation coefficients (*R*) across the 55 samples for the 24 (of 48) pairs with expression data for both the retrocopy and the parental gene.

Results

The Number, Age, and Structure of Retrocopies

We began with 46,235 nonredundant rice protein sequences and defined 19,235 of them as potential parental genes based on intron and coding characteristics (see Materials and Methods). Mapping this group of potential parental genes to the *japonica* genome, we identified 150 retrocopies that arose from 143 parental genes (supplementary table S1, Supplementary Material online). None of the retrocopies contained introns, and the vast majority (93%) lost ≥2 introns relative to their putative parental genes. Of the 150 retrocopies, 147 contained target site duplications (TSDs) of 7–60 bp repeats within 500 bp upstream or downstream; TSDs are a common by-product of the retroposition process (Vanin 1985) but may also simply be an inherent sequence characteristic. In addition, 21 retrocopies possessed poly-A tails, which is another strong indicator of retroposition.

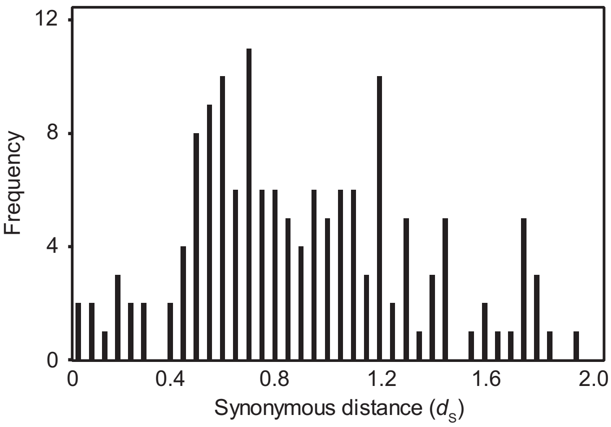


Fig. 1.—The distribution of the number of synonymous substitutions per site (d_S) for the 150 retrocopies compared with their parental genes.

We estimated the number of synonymous substitutions per site (d_S) between each retrocopy and its parental gene. d_S values ranged from 0.0 to 2.0, but few pairs had d_S values < 0.4 (fig. 1). The frequency distribution of d_S featured a peak at ~0.7, corresponding to a divergence time of ~54 Myr based on a rate estimate of 6.5×10^{-9} substitutions per site per year in the grasses (Gaut et al. 1996). These results imply that the retrogenes are considerably older than the 0.44 Myr divergence between *indica* and *japonica* (Ma and Bennetzen 2004). To verify this implication, we compared the 150 retrocopies against the *indica* genome sequence using BlastN; 137 of the retrocopies were shared between *japonica* and *indica* with ≥90% nucleotide identity and ≥70% sequence coverage. Another 12 retrocopies were located within insertions in *japonica* relative to *indica*, and one retrocopy was not found in *indica*.

The age of retrocopies suggests they may be functionally conserved. This suggestion is supported by estimates of the ratio of nonsynonymous to synonysmous changes (ω) between retrogenes and parental genes. Mean $\omega(\bar{\omega})$ for the 150 retroparent gene pairs was 0.51 (supplementary table S1, Supplementary Material online), which is far less than the neutral expectation of ω = 1.0. For individual gene pairs,

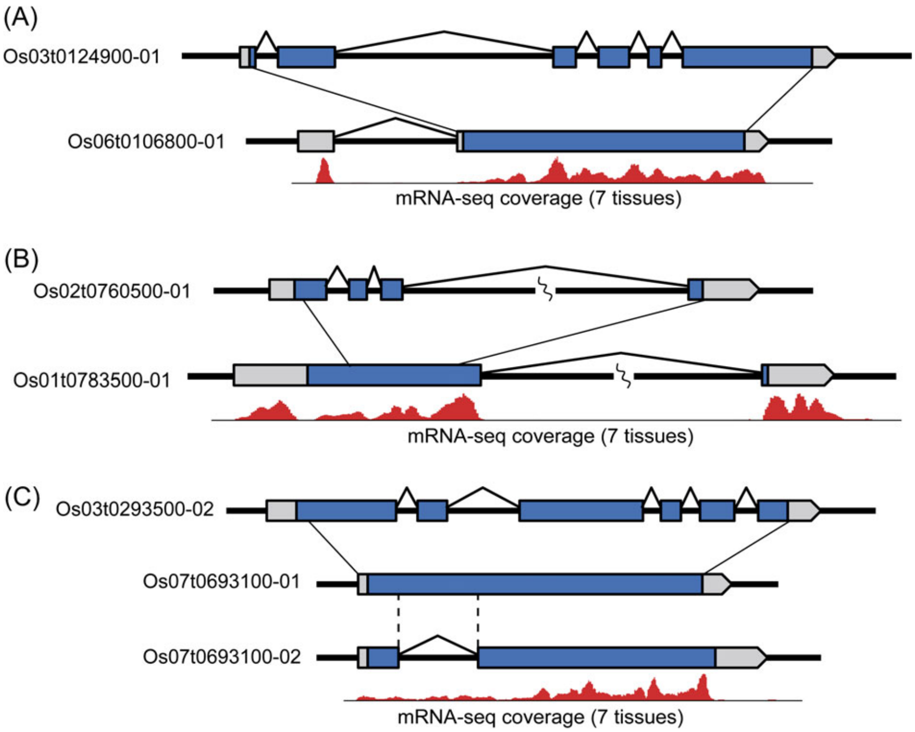


Fig. 2.—Examples of retrogenes with altered structures compared with their parental genes. (*A*) The retrocopy (bottom) has evolved a new 5′ UTR relative to its parental gene (top). (*B*) The retrocopy (bottom) evolved a new 3′ exon consisting of a part of protein coding region. (*C*) An example of an intronization event. There are two cDNA variants in the retroposed region (Os07t0693100-01 and Os07t0693100-02). Part of the Os07t0693100-01 transcript is missing in Os07t0693100-02, presumably due to an intronization event. In all figures, the blue boxes indicate annotated protein coding regions and the gray boxes indicate UTRs. Red histograms under the retrogene structures diagram the numbers of mRNAseq reads associated with the retrogenes.

123 of 150 (82%) had an estimated ω significantly <1.0 at P value < 0.01 (supplementary table S1, Supplementary Material online).

Despite this conservation, the 150 retrocopies did encompass structural changes relative to their parental genes. For example, 90 of the 150 retrocopies contained either a premature stop codon or a frameshift replacement. These 90 have a significantly higher $\bar{\omega}$ value (0.56) than the 60 intact retrocopies ($\bar{\omega}$=0.44). The difference in $\bar{\omega}$ is significant (Wilcoxon rank sum [WRS] test, $P < 10^{-3}$), suggesting that intact and modified retrocopies differ in levels of constraint. In addition, eight retrocopies contributed to modified gene structures (fig. 2 and supplementary table S1, Supplementary Material online). Of these, six evolved new 5′-exons or untranslated regions (UTRs) and two added 3′-exons. Seven of the eight new structures were confirmed by full-length cDNA (flcDNA) sequences from rice, and the remaining retrocopy matched an flcDNA from maize. In addition, flcDNAs indicated potential intronization in three additional retrocopies. In these copies, a portion of the coding region of the parental gene apparently became an intron after retroposition (fig. 2 and supplementary table S1, Supplementary Material online). However, none of the three obey the canonical GT/AG rule for splice sites. For one of the three loci (fig. 2), there were flcDNA variants with and without the intron, suggesting that the newly emerged intron is unstable and not constitutively spliced.

Expression Divergence between Retrogenes and Their Parental Genes

To investigate the transcriptional activity of the 150 retrocopies and their parental genes, we sampled seven tissues of rice with mRNAseq. In total, we generated 278,060,869 sequence reads, of which 60% (166,511,914) were uniquely mapped on the *japonica* genome (table 1). The mapped reads comprised ~7.8 Gbp in total and ranged from 887 Mbp to 1.4 Gbp among the seven tissues. On average, our mRNAseq data resulted in ~21-fold coverage of the rice transcriptome for each tissue (table 1).

We further assessed coverage by examining expression in the 22,973 loci that are both supported by rice flcDNAs (Kikuchi et al. 2003) and unrelated in sequence to TEs. By applying a background level determined from intergenic regions (see Materials and Methods), we determined that 22,279 (or 97.0%) of the loci were expressed in one or more tissues. Broadening the gene sample to include ab initio predicted genes, we detected expression in 32,164 (76.9%) of 41,847 non-TE–related loci. These results

Table 1
Number of Short-Reads Derived by RNAseq and Summary of Mapping Results

Tissue	Total No. of Short Reads	No. of Uniquely Mapped Reads	Total Nucleotide Length of the Mapped Reads (bp)	Fold Coverage of the Uniquely Mapped Reads against RAP-Annotated Regions
Callus	36,642,482	23,506,559	1,071,781,348	20.2
Leaf	33,537,231	19,334,815	886,753,671	16.7
Panicle (before flowering)	39,438,983	22,845,156	1,051,085,731	19.8
Panicle (after flowering)	46,071,816	28,541,187	1,392,167,801	26.2
Root	33,307,824	20,493,666	927,208,012	17.5
Seed	48,619,562	27,646,800	1,364,148,154	25.7
Shoot	40,442,971	24,143,731	1,135,190,328	21.4
Total	278,060,869	166,511,914	7,828,335,045	147.4

suggest that our mRNAseq data are sufficient to examine the rice transcriptome, including retrocopies.

Of our 150 retrocopies, 100 were expressed above the background level in one or more tissues; we deem these 100 transcribed retrocopies as retrogenes. Of the remaining 50 retrocopies, 32 were covered by one or more mRNAseq reads or by other transcriptional evidence, such as cDNA and expressed sequence tags (ESTs), which suggests that these retrocopies could be transcribed either at levels below the detection limits of our study or in tissues not included in our study. The remaining 18 retrocopies had no transcriptional evidence from any data source.

We sought to compare expression patterns between the 100 retrogenes and their parents, but 13 parental genes had no expression in our mRNAseq data. For the remaining 87 gene pairs, we investigated patterns and levels of expression. For expression level, we calculated the maximum number of RPK (mRPK) among the seven tissues for each gene. The mean mRPK value of the retrogenes (880.2) was significantly lower than that of parental genes (3,283.9) (WRS test, $P < 0.005$). Parental genes were also expressed at significantly higher levels, on average, than all other expressed, non-TE–related genes (1,5940.0; WRS test, $P < 10^{-3}$). In contrast, mean mRPK values did not differ between retrogenes and all other non-TE–related genes ($P > 0.1$). Thus, parental genes are expressed at significantly higher levels than the genomic average.

To compare expression patterns between a retrogene and its parental gene, we calculated the Pearson product-moment correlation coefficient (R) between genes, using observed RPK values from the seven tissues. Thirty-eight of the 87 retroparent pairs exhibited slightly or strongly negative correlations, with the most negative being -0.58 (supplementary table S1, Supplementary Material online). However, 56% had $R > 0.00$, and some R values were strongly positive (supplementary table S1, Supplementary Material online). For example, ten retrocopies had R values > 0.90, and an additional 31 pairs had R values > 0.50. We graphed expression values for some of the highly correlated pairs (fig. 3), visually verifying that these retrogenes are expressed at lower levels than their parental genes but in a similar pattern across tissues.

Given the correlation in gene expression between retrogenes and their parental genes, we next investigated the relationship between R and evolutionary time, as measured by molecular divergence (d_S). To do this, we followed precedent and first transformed R by the equation $Y = \log((1 + R)/(1 - R))$ (Gu et al. 2002; Makova and Li 2003; Li et al. 2009). Plotting Y against d_S produced a clear negative correlation (fig. 4; $R = -0.30$, $P = 0.005$), implying that newer retrogenes are expressed more like their parental genes than are older retrogenes. To verify the significance of the negative correlation (fig. 4), we bootstrapped samples from the original data and calculated R for each of 10,000 resampled data sets. The resulting distribution was significantly negatively skewed, and the 95% confidence intervals (CIs) did not include zero. In fact, only 19 (0.2%) of the 10,000 resampled data sets had a correlation coefficient ≥ 0.0 (supplementary fig. S1, Supplementary Material online). To sum, the expression of retrogenes and parental genes tend to be positively correlated, and this correlation degrades as a function of evolutionary time, as measured by d_S.

Expression Divergence in DNA- and RNA-Based Duplicates

Previous studies have established negative correlations between Y and d_S for DNA-based duplicates (Gu et al. 2002; Makova and Li 2003; Li et al. 2009). Given that our RNA-based duplicates exhibit a similar relationship, we sought to determine whether the dynamics of expression divergence differ substantially between RNA-based and DNA-based duplicates. To identify pairs of DNA-based duplicates for this analysis, we defined gene families and culled independent pairs of genes from these families (see Materials and Methods; Makova and Li 2003), resulting in 3,420 gene pairs. We then estimated d_S between pairs of duplicated genes and measured Y between duplicates using our mRNAseq data.

The analysis of DNA-based duplicates yielded two notable observations. First, the mean of $Y(\bar{Y})$ across 3,420

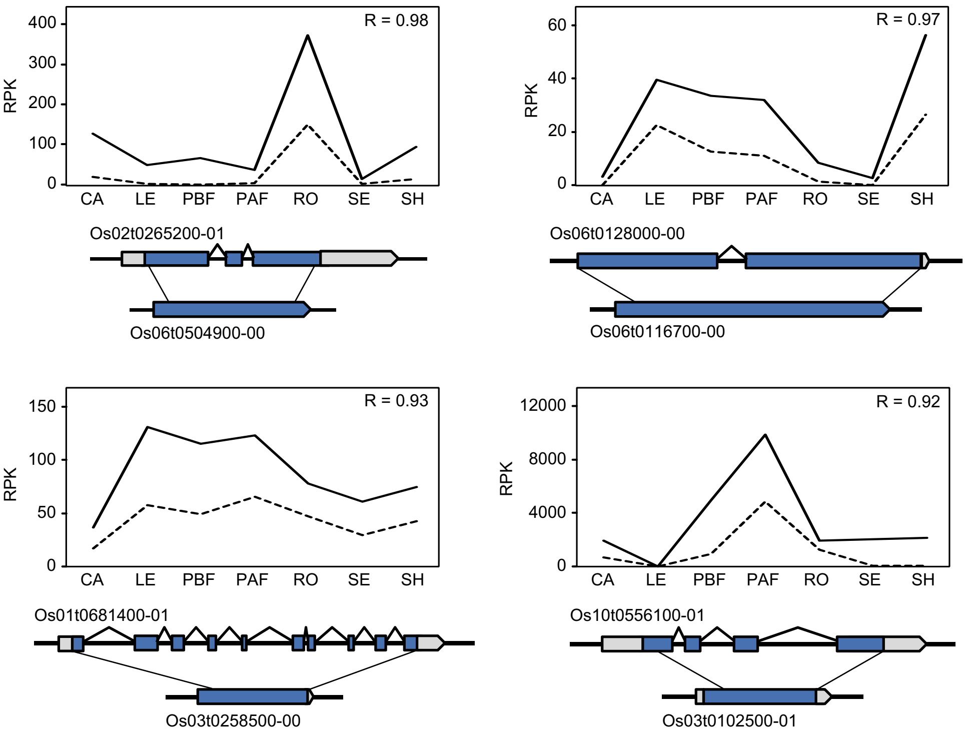
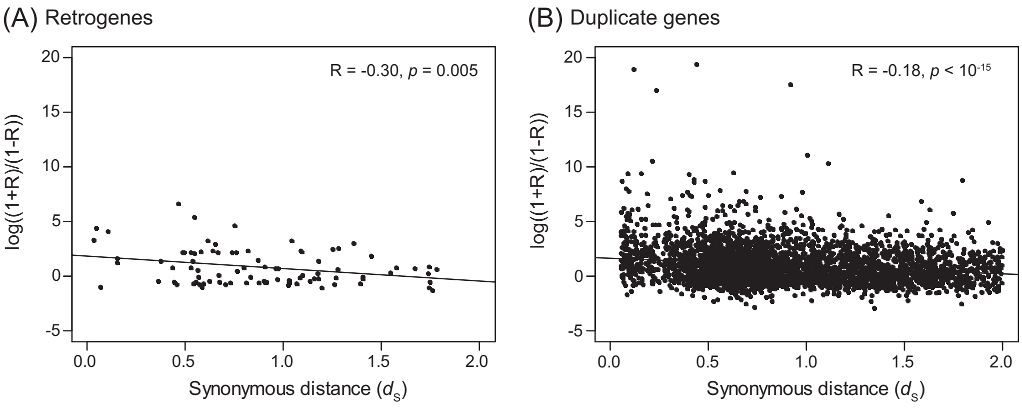


Fig. 3.—Diagram of gene expression counts for the parental gene (solid line) and the retrogene (dashed line) in seven tissues (CA = callus, LE = leaf, PBF = panicle before flowering, PAF = panicle after flowering, RO = root, SE = seed, SH = shoot). The diagrams beneath each graph represent the structure of the parental gene (top) and its retrocopy (bottom).

DNA-based duplicates was 0.99 compared with $\bar{Y}$=0.81 for the 87 RNA-based duplicates. However, this difference was not significant (WRS test, $P > 0.1$). To assess whether these $\bar{Y}$ values were significantly different from random noise, we randomly selected 87 and 3,420 pairs of genes from the set of 32,164 non-TE–related genes and calculated $\bar{Y}$ for these data sets. After repeating the analysis 10,000 times, we found that $\bar{Y}$ values of 0.81 and 0.99 were much higher than the random expectation ($P < 10^{-3}$ and $P < 10^{-4}$, respectively, supplementary fig. S2, Supplementary Material online). Second, Y and d_S were negatively correlated for the DNA-based duplicates ($R = -0.18$; $P < 10^{-15}$), as they were for the RNA-based duplicates (fig. 4). To assess whether the correlations in figure 4*A* and *B* were statistically different, we subsampled from the pairs of 3,420 DNA-based duplicates to produce 10,000 data sets of 87 gene pairs. We then calculated the correlation R between Y and d_S for each of these data sets to estimate a CI. The interval include −0.30, indicating that the correlation between Y and d_S is statistically indistinguishable ($P = 0.12$) and suggesting


(A) Retrogenes

(B) Duplicate genes

Fig. 4.—The relationship between the correlation in gene expression between pairs of genes, as represented by Y [=log((1 + R)/(1 − R))], and molecular divergence (d_S). (*A*) RNA-based duplicates (Retrogenes). (*B*) DNA-based duplicates.

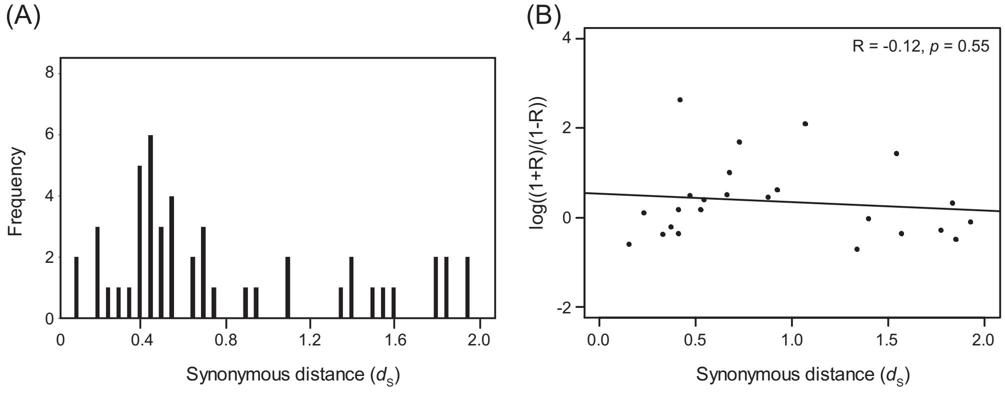


Fig. 5.—Analyses of retrocopies from *Arabidopsis thaliana*. (*A*) The distribution of d_S between 48 retrocopies and their parental genes. (*B*) The relationship between Y [=log((1 + R)/(1 − R))] and molecular divergence (d_S) for 24 retroparent pairs with available expression data.

that gene expression diverges at similar rates for RNA- and DNA-based duplicates.

Promoter Expression and Divergence

What might cause the correlation in expression between a retrogene and its parent? One possible mechanism is the co-retroposition of promoter sequences with coding regions (Okamura and Nakai 2008). If promoter regions are retroposed, the retrogene and its parental gene would be expected to initially have similar expression patterns.

We assessed the probability of this mechanism in two ways. First, we investigated whether the promoter region of parental genes is transcribed. We limited the analysis to 52 parental genes that were validated by rice flcDNAs and also had 500 bp upstream sequences that did not overlap with other genes. For these 500 bp regions, the average RPK summed across tissues was 124, which is higher than the average summed RPK (70) of the 1,885 intergenic regions (see Materials and Methods and supplementary table S2, Supplementary Material online). Thus, there is evidence for leaky transcription of the promoter regions of parental genes, suggesting that retroposition of promoters is possible. However, the parental genes have no obvious tendency toward aberrantly leaky expression of regulatory regions compared with other genes because the average expression of 500 bp upstream regions was slightly higher (148 RPK) for a sample of 15,480 nonparental genes.

Second, we examined upstream sequences to search for sequence similarity between retrogenes and parental genes. There were 36 pairs in which both retrogene and parental gene were validated by rice flcDNAs and had 500 bp 5′ regions that did not overlap with adjacent genes. For these 36 pairs as well as 2,602 validated pairs of DNA duplicates, we examined divergence of the 500 bp upstream sequences with d_{SM} (Castillo-Davis et al. 2004). The d_{SM} metric varies from 0–1, with higher values representing greater divergence in motifs. By this metric, upstream sequences of retrogene pairs (mean $d_{SM} = 0.63$) were more highly diverged than those of duplicate gene pairs (mean $d_{SM} = 0.58$; WRS $P < 0.05$). Moreover, the d_{SM} values for retrogene pairs did not differ significantly from pairs of rice genes chosen at random from throughout the genome (data not shown); in other words, as a group, the upstream sequence of retroparent pairs were no more closely related than randomly chosen genomic regions.

Expression and d_S Analyses in *A. thaliana*

To begin to assess the generality of our observations, we analyzed expression correlations and molecular divergence in *A. thaliana* retrocopies (Zhu et al. 2009). (Retrocopies have also been characterized in poplar [Zhu et al. 2009], but only *A. thaliana* has sufficient genomic resources to analyze expression at this time.) We first identified the parental genes of 69 retrocopies previously identified by Zhang et al. (2005) and then estimated d_S between retroparent pairs. The resulting distribution of d_S revealed a peak encompassing d_S ~0.4 to ~0.6, with few young retrocopies (fig. 5). We also compared expression patterns between retrogenes and parental genes using microarray-based expression data (Schmid et al. 2005; Matsuda et al. 2010). For the 24 retroparent pairs on the microarray, $\bar{Y}$ was 0.36, a value significantly larger than mean values derived from randomly generated data sets ($P < 0.05$). In additional, Y was negatively correlated ($R = -0.13$) with d_S (fig. 5). The correlation was not significant ($P > 0.1$), but the lack of significance may be due to low sample size ($n = 24$).

Discussion

We detected 150 retrocopies in the *japonica* rice genome, which is far fewer than the 1,235 identified by Wang et al. (2006). The discrepancies between studies is due in part to the more stringent criteria applied in our study. First, we retained only the homologous hits between parental and retrogenes that yield $d_S < 2.0$; 662 of 1,235 of the retrocopies from Wang et al. (2006) fell into this category. Second, we screened carefully for TE-related sequences (see Materials and Methods), to avoid possible misinferences due to misannotation or TE activity. Reanalyzing the data of Wang et al. (2006)—which was based on earlier less complete genome annotations—we found that parental cDNA sequences of 216 of the 662 retrocopies with $d_S < 2.0$ contained masked repetitive sequences. Third, our pool of potential parental genes was more stringent. For example, we disallowed the use of genes that had only one intron and a downstream poly-A tract; more than 150 of the parental genes of the 446 non-TE–related retrocopies from Wang et al. would have been disregarded on this basis. Fourth, we did not allow retrocopies and their source genes to be tandemly duplicated because retrocopies are expected to integrate randomly in the genome rather than proximately (Zhang et al. 2002). In contrast, 126 of the Wang et al. non-TE–related retrocopies are within 100 kb of their parental gene; most of these pairs have low d_S (<0.4) values, suggesting that gene conversion, which occurs more often between genes on the same chromosome (Mondragon-Palomino and Gaut 2005), may have affected evolutionary divergence. Finally, our homology criteria were stricter. We required ≥35% identity over ≥70% of the parental genes, whereas Wang et al. (2006) did not set an identity threshold.

Given that careful searches of the *A. thaliana* genome identified only 69–83 retrocopies (Zhang et al. 2005; Zhu et al. 2009), our total of 150 retrocopies seems reasonable for the ~3-fold larger rice genome. Although our total estimated number of retrocopies in the *japonica* genome could be conservative, the estimate of 1,235 from Wang et al. (2006) may have been, in retrospect, too liberal.

Rice Retrocopies Tend to be Old

The d_S distribution for our retrocopies produces a peak of divergence in the range from ~0.5 to 0.9 (fig. 1). Assuming that synonymous substitutions are neutral and evolve at a rate similar to other genes (Gaut et al. 1996), this d_S distribution corresponds to insertion times of ~40 to 70 Myr. This age distribution is surprising, for two reasons. First, our conservative approach should be biased toward identifying fairly new retrocopies that have had little time to diverge from their parental genes. Second, if retrocopies are generated and eliminated in a constant manner, then younger retrocopies should be more frequently observed than older retrocopies (Lynch and Conery 2000). Taken together, the d_S distribution suggests that the frequency of successful retroposition events was higher ~40 to ~70 Ma than in the more recent past.

The salient question is "Why?" We can think of two possibilities, neither of which can be proven at this time. The first is that the retroelements responsible for retroposition have decreased in activity over the last ~50 Myr. Clearly some retrotransposons have been active recently. For example, many long terminal repeat (LTR) retrotransposon insertions are <~3 Myr old (Wicker and Keller 2007; Baucom et al. 2009). However, it is difficult to conclude from this information whether LTR activity has decreased, increased, or remained the same for ~50 Myr because older insertions are expected to be deleted rapidly from the rice genome (Ma and Bennetzen 2004). In mammals, gene retroposition is often attributed to long interspersed nuclear elements (LINEs) (Esnault et al. 2000). The extant rice genome has very few LINEs, and LINE transcriptional activity is relatively weak compared with other TEs in the rice genome (Jiao and Deng 2007). If LINEs were more active in the past, it may explain the peak of old retroposition events.

The second possibility is that successful rice retroposition events are associated with a period of genomic flux following a whole genome duplication (WGD) event ~50 to 70 Ma (Paterson et al. 2004; Schlueter et al. 2004; Fawcett et al. 2009). The *A. thaliana* data provides (weak) support for this interpretation because the d_S peak from ~0.4 to 0.6 (fig. 5) corresponds to a date of 20–30 Myr (Koch et al. 2000), and this range encompasses the earliest dates estimated for the most recent polyploidy event in the *A. thaliana* lineage (Beilstein et al. 2010). Importantly, the two possibilities—retrotransposon activity and WGD events—may be related because polyploidy has long been recognized as a genomic stress that could promote TE activity (reviewed in Tenaillon et al. 2010).

Patterns of Retrogene Expression

Two-thirds of the 150 rice retrocopies are expressed in our mRNAseq data. This is probably an underestimate of the true proportion of expressed retrocopies because the number of tissues (7) and experimental conditions (1) were limited. Indeed, another 32 of the 150 retrocopies have some mRNAseq reads and/or EST evidence suggesting they may be expressed. The total proportion of expressed retrocopies in rice (66–88%) contrasts starkly with human retrocopies, of which only 30% are expressed (Vinckenbosch et al. 2006). One has to be careful with comparisons across studies because different studies use different methods to identify retrocopies and to assess expression. Nonetheless, the contrast between rice and humans contributes to the overarching impression that the proportion of expressed rice retrocopies is relatively high.

Studies in human and fruitflies have shown that retrogenes are expressed in more tissue-specific manners than their parental genes, and several retrocopies have evolved testis-specific expression (Marques et al. 2005; Vinckenbosch et al. 2006; Bai et al. 2007). Our rice data paint a less clear picture. For example, our retrogenes are expressed in significantly fewer tissues than their parental genes (4.8 vs. 5.6 tissues; WRS $P < 0.05$), but the retrogenes as a group are neither more tissue-specific (table 2) nor exhibit lower

Table 2
Comparison of the Number of Tissue Specific Genes in Each Tissue between Retrogenes and Parental Genes

	Retrogene		Parental Gene		
Tissue	No. of Tissue-Specific Genes[a]	No. of Nonspecific Genes	No. of Tissue-Specific Genes[a]	No. Nonspecific Genes	*P* Value
Callus	15	72	17	66	0.60
Leaf	13	74	20	63	0.13
Panicle 1[b]	24	63	27	56	0.48
Panicle 2[b]	26	61	32	51	0.24
Root	34	53	26	57	0.29
Seed	13	74	12	71	0.93
Shoot	22	65	14	69	0.18

[a] Tissue-specific genes are defined based on Ueda's Akaike's Information Criterion–based method.
[b] Panicle 1, panicle before flowering; panicle 2, panicle after flowering.

Shannon entropies (a measure of the breadth of expression) than their parental genes (data not shown).

However, both expression patterns and some structural characteristics hint that retrogenes could be filling new functions. For example, 29 of our 87 retrogenes are expressed in a new tissue—that is, a tissue in which their source gene is not expressed. Similarly, 13 parental genes were not expressed in our data, when their retrocopy was expressed. With regard to structure, full-length cDNA suggest that some retrocopies contain new exons and introns (fig. 2). The intronization of exons has been identified previously in retroposed genes of mammals (Szczesniak et al. 2011), but the functional implications of intronization are not yet known.

Because retrogenes are typically thought to acquire new regulatory regions, our a priori expectation was that expression patterns would be completely uncorrelated between retrogenes and parental genes. In contrast to our expectation, expression for most retroparent pairs is positively correlated (fig. 4 and supplementary table S1, Supplementary Material online) and sometimes strongly so (fig. 3). Moreover, expression diverges as a function of molecular divergence (d_S) at a rate statistically indistinguishable from that of DNA-based duplicates (fig. 4; Li et al. 2009). Our power to distinguish differences may be limited due to small sample sizes of both retrocopies and tissues, but the similarity in expression divergence between RNA- and DNA-based duplicates is surprising nonetheless.

One possible mechanism underlying expression correlation between a retrogene and its source is the co-transcription and co-retroposition of 5′ promoter sequences along with coding regions (Okamura and Nakai 2008). Consistent with this mechanism, we found that upstream sequences of parental genes are, indeed, transcribed above background levels. However, parental genes have lower levels of upstream expression than the genomic average. In addition, our analysis of sequence similarity in upstream regions yielded little evidence for homology; by the measure d_{SM}, retrogene and parental gene are no more closely related in sequence than pairs of genes taken at random from throughout the genome. Our results are similar to those from *Drosophila*, for which there was no clear sequence homology in promoter regions between retrogenes and their source (Bai et al. 2008). Based on these observations, it seems unlikely that the most common cause of correlated expression is retroposition of 5′ regulatory regions from the parental gene.

This leaves three possibilities for the genesis of retrogene regulation: 1) that retrogenes acquire de novo regulatory elements, 2) that retrogenes use nearby genes to drive expression, or 3) that regulatory elements are embedded within retroposed regions, such as exons and UTRs. Each of these may be true to some extent. For example, previous studies have shown that retrogenes tend to be located near other genes (Vinckenbosch et al. 2006), suggesting that nearby genes drive retrogene regulation. Similarly, the 100 rice retrogenes expressed in mRNAseq data are significantly closer (mean = 3.7 kb) to annotated genes than the 50 nonexpressed retrocopies (mean = 7.7 kb) (WRS test, $P < 0.01$). Yet, the acquirement of nearby promoters is unlikely to produce correlated expression patterns. Of these three possibilities, only one—embedded regulatory elements within the transcribed region—provides a mechanism to explain the correlated patterns of expression between retrogenes and parental genes.

The Characteristics of Successful Retroposition Events in Plant Genomes

It seems likely that the retrocopies identified in rice and *A. thaliana* by computational approaches represent only a subset of retroposition events because most events are probably lost quickly to evolutionary history. Our studies provide information about the general characteristics that may make them successful. First, in rice, these events are most likely to arise from genes that are highly expressed. Second, they are biased for events that lead to similar expression patterns, at least initially, between the retrogene and its source. Retrocopies with similar expression may be favored because altered expression patterns usually confer deleterious effects. (As an extreme example, ectopic expression of homeobox genes results in abnormal leaf development

[Matsuoka et al. 1993; Lincoln et al. 1994; Muehlbauer et al. 1999]). The bias to retain events with correlated expression patterns may favor the retroposition of genes that have embedded regulatory elements or leaky transcription from upstream regions, although we believe the latter to be uncommon. Third, successful events tend to occur at certain times in genomic history. It is not hard to imagine that RNA-based gene duplication events may be more successful at a time when DNA-based duplications are also rampant—for example, during the aftermath of a WGD event.

Supplementary Material

Supplementary figures S1 and S2 and Supplementary tables S1 and S2 are available at *Genome Biology and Evolution* online (http://www.gbe.oxfordjournals.org/).

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