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Evidence for an evolutionary force that prevents epigenetic silencing between tail-to-tail rice genes with a short spacer☆

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Abstract

During the course of evolution, the genome should have toned down various types of genomic noise, such as those that cause the unstable expression or gene silencing observed in transgenic organisms. We found a rice genomic segment where two genes, encoding 5-enolpyruvylshikimate-3-phosphate synthase (*EPSPs*) and ribosomal protein small subunit 20 (*rps20*), are located in a tail-to-tail orientation and separated by only 300 bp of spacer. It is possible that this kind of structure would give rise to unstable expression due to antisense RNA derived from the neighboring gene. We examined this possibility using Northern blot, reverse transcription-polymerase chain reaction (RT-PCR), and 3′ RACE analyses, but obtained no evidence for instability or antisense RNAs of these housekeeping genes. Comparison of the sequences in the corresponding regions among related rice species revealed a lower level of genetic divergence of both the 3′-untranslated region (3′-UTRs) than of the other noncoding regions; in particular both of the boundaries between the 3′-UTRs and the spacer were markedly conserved. The conservation of both the terminal regions is most likely the result of purifying selection, implying a functional role for the strict termination of the transcription of these genes to prevent gene-silencing-related events.

Keywords: Endogenous rice genes; Evolutionary force; Gene silencing; Gene expression stability; 3′-UTR

1. Introduction

Genomic structures have established order among their components to confer stability on gene expression during evolution (Ludwig, 2002). Unlike exogenous genes, the endogenous genes should have evolved so as to restrain instability. However, we do not have substantial evidence about how the genome prevents disturbances arising from neighboring endogenous genes. In the genome context, noncoding regions and intergenic spacer regions seem to profoundly influence gene expression, although the function of these regions remains poorly understood (Shabalina et al.,

Abbreviations: EPSPs, 5-enolpyruvylshikimate-3-phosphate synthase; rps20, ribosomal protein small subunit 20; 3′-UTR, 3′-untranslated region; PTGS, posttranscriptional gene silencing; wx, waxy; CV, coefficient of variation; dsRNA, double-stranded RNA; RT-PCR, reverse transcription-polymerase chain reaction; ND, number of nucleotide differences per site; indel, insertion and deletion; I, incidence of indel; S, size after excluding all the indels; I/S, indel incidence index.

☆ Nucleotide sequences; the accession number AB052962 (EPSPs cDNA) and AB124881-AB124893 (*EPSPs–rps20* genomic region in 13 strains of the *Oryza* AA genome species).

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2001). Decoding genomic sequences alone is not enough to reveal the yet undeciphered functional elements and mechanisms of the elaborate genome structure. Interspecies comparison of detailed genomic sequences is one of the most powerful ways to demonstrate that a segment of DNA is functionally important (Waterston et al., 2002).

Most of the genes of various model species, including rice, are now being identified as a result of the decoding of the genomic sequences. These sequencing projects account for the detailed organization and position of genes residing on each chromosome. However, we do not know whether or not genes are arranged at functionally meaningful and optimal positions in the genome, although the elements in the genome have surely evolved in a fashion ensuring that ordinary gene expression occurs without a hitch. On the other hand, many transformed organisms show irregular expression or silencing phenomena of their exogenous genes. From the nucleotide sequences of rice chromosome 1 (Sasaki et al., 2002), at least 88 sites of unusual gene organization such as genes overlapping on the different strands and tail-to-tail-oriented genes with less than 1000-bp spacers have been found (as of August 2004 at http://www.shigen.nig.ac.jp/rice/oryzabase/genome/chromosomeList.jsp#chr1). These genes have the potential to interfere with normal expression of the adjacent gene: if a transcript invades the neighboring gene or extends beyond the spacer, the result may be interference with the expression of each gene via antisense RNA or posttranscriptional gene silencing (PTGS) mechanisms. Thus, these genes should be regulated in various ways to maintain normal function. As demonstrated in a human genetic disease, antisense RNA resulting from the juxtapositioning of neighboring genes due to a deletion mutation results in abnormal gene regulation (Tufarelli et al., 2003).

We have surveyed the genomic structure around the rice *waxy* (*wx*) gene and found two housekeeping genes encoding 5-enolpyruvylshikimate-3-phosphate synthase (*EPSPs*) and ribosomal protein small subunit 20 (*rps20*) in a region about 45 kb downstream of *wx* (Sato et al., 1999). The *EPSPs* and *rps20* genes are adjoined with only a 303-bp spacer, and are directed in a tail-to-tail orientation. Here we have focused on these genes because these are indispensable in plants, and their normal function must have been maintained despite the potential instability caused by antisense RNA or PTGS.

We show here that these *EPSPs* and *rps20* genes were expressed in most of the organs of rice plants. Their transcriptional termination was strictly restricted within a small range, and no transcripts beyond the spacer were detected. We were interested in seeing whether or not the region between these genes, including both 3′ end regions and the spacer, has an evolutionary role in counteracting the potential instability of the gene expression. Further analysis was performed to examine the sequence conservation of the terminal regions of the two genes. The nucleotide sequences of the exon, intron, 3′-untranslated region (UTR) and spacer regions in the *EPSPs–rps20* fragment were compared among 14 related rice strains. The comparison revealed conservation of both the 3′-untranslated region (3′-UTRs), particularly both of the boundaries between the 3′-UTRs and the spacer. Our findings suggest that secure transcriptional termination is the basis of one of the mechanisms preventing epigenetic silencing of tail-to-tail-oriented genes with a small spacer, such as the rice *EPSPs–rps20* genes.

2. Materials and methods

2.1. cDNA preparation from rice calli and screening

As described by Yoshida et al. (1994b), an initial callus was induced from Japonica seed (*Oryza sativa* cv. Yamahoushi) on MS medium containing 2,4-D at 2 mg/l. One-month-old calli were then transferred to suspension culture and maintained in R2 medium (Ohira et al., 1973) supplemented with 2,4-D at 2 mg/l. Total RNA was isolated from 8-week-old calli which were subcultured every week (Yoshida et al., 1994a). The SDS–phenol method employed by Watanabe and Price (1982) was applied for RNA extraction, and RNA was precipitated with lithium chloride. Poly(A)-RNA was purified using a magnetic separation system with a biotinylated oligo(dT) probe and Streptavidin MagneSphere Paramagnetic Particles (Promega, Madison, WI). Synthesis of cDNA from the callus mRNA was performed with a cDNA synthesis kit (Time Saver cDNA Synthesis Kit: Amersham Biosciences, Buckinghamshire, UK). An *Eco*RI–*Not*I–*Bam*HI linker was ligated to both ends of synthesized cDNAs, and excess linker was removed using a Spin column (Amersham Biosciences). The obtained cDNA was cloned into lambda Zap II vector (Stratagene, La Jolla, CA). Packaging was performed according to the manufacturer's protocol (Amersham Biosciences). The cDNA clones for the *EPSPs* and *rps20* genes were screened by using a specific PCR probe for each gene as described below. The probes were prepared using the following primers:EPSPs: 5′TGGCTTCCTGGAGAGTAAAGGA3′+5′TTCTGAAA-′T T C T G A A A T C G C A G A A G G C A 3′r p s 2 0 : 5′CAAGGTGCTCCACATCACCAC3′+5′GTTCCACCA-′GTTCCACCAGTGTTCTCATGCTA3′Plaque hybridization was carried out using the ECL gene detection system (Amersham Biosciences).

2.2. Northern blot hybridization

Total RNAs were isolated from roots, seedlings, spikelets, and anthers of Japonica (T65*wx* line) and Indica (PTB10 line) rice, respectively. Twenty micrograms of each RNA preparation was electrophoresed on a 1% agarose gel containing formaldehyde, and transferred onto a nylon membrane (Positively Charged, Roche, Basel). The cDNA clones of the *EPSPs* and *rps20* genes were employed as