A Novel Approach Obtaining Intron-Containing Hairpin RNA Constructs

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Recently, much research on constitutive expression of an intron-containing self-complementary hair-pin RNA (ihpRNA) have been reported to silence target genes efficiently in a variety of species. Here we designed a new recombinant-PCR mediated method called direct amplification of ihpRNA from genomic DNA. This approach has proved to be easy, stable, and efficient.

Key words: intron-containing hairpin RNA; genomic DNA; recombinant-PCR; Zea mays; auxin-binding protein

RNA-mediated gene silencing is a conserved mechanism that recognizes dsRNA as a signal to trigger sequence-specific degradation of homologous mRNA. It has been used as an efficient tool in gene-function exploration and gene engineering in a variety of organisms.1–3 Recent discoveries indicate that ihpRNA construction is an extremely effective triggering signal of gene silencing. For example, in Smith’s experiment, almost 100% of plants transformed with an intron-containing hairpin RNA construction showed silencing.4 The presence of a spliced intron in the transgene encoding the dsRNA appears to enhance silencing efficiency.5

To generate ihpRNA constructs, several methods have been used. For instance, a generic vector ‘pHANNIBAL’ with a functional intron has been reported.5 Sense and anti-sense PCR products were cloned into the locations flanking the intron, which require several rounds of restriction and ligation. Another simpler method applying panhandle-PCR that directly amplifies ihpRNA constructs from genomic DNA has been proposed.6 Unfortunately, we failed to amplify the right sequence following their protocol several times, due to a suppressive PCR effect. Hence, we developed a recombinant-PCR7 mediated method called direct amplification of ihpRNA from genomic DNA. The resulting ihpRNA could be cloned into any expression vector bidirectionally besides of its stability and efficiency.

Our approach is described in Fig. 1. Since the final product was two inverted repeats of an exon with an intron between them, nothing could be better than genomic DNA or cloned genomic DNA as the template. Appropriate primers played a key role in efficient amplification of the target fragments. Two sets of primers are designed to amplify the intron-exon and the exon sequences respectively. Primer 1 contained a 5′ heel reverse complementary to the 5′ end of exon B. Primer 2 showed the opposite characteristic, in that it

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Abbreviations: dsRNA, double-stranded RNA; hnRNA, heterogeneous nuclear RNA; hpRNA, hairpin RNA; ihpRNA, intron-containing hairpin RNA; PTGS, post-transcriptional gene silencing; RM-ihpRNA, recombinant-PCR mediated ihpRNA constructs generating method; RNAi, RNA interference
was completely reverse complementary to primer 1. The intron-exon fragment and the exon itself were amplified in two different tubes simultaneously. After the first PCR process was finished, the two products became new templates and annealing, through their complementary parts, then extended, and finally the fused sequence formed. In later cycles, the fused sequence increased exponentially with primer 3 working as both sense and anti-sense primer. Thus, the ihpRNA construct was achieved.

The gene coding auxin binding protein from Zea mays (Zm-abp1; GenBank accession no. L08425) was first used as the original template for the ihpRNA construct, which contained two inverted repeats of part of Zm-abp1 exon 3 and its upstream intron as the spacer. The gene map of Zm-abp1 is shown in Fig. 2. The exons are underlined, and the parts primers were designed from are shadowed. Most of the noncoding sequence shown by dots is omitted, because the full sequence was too long. The locations of exons and primers are marked.

The reason the inclusion of an intron in these constructs had a consistently enhancing effect is still unclear. Supposedly, the intron cutting process makes the sense and anti-sense fragments match each other better. Other researchers think that this construct can be spliced by spliceosome or that the intron-cutting process generates a small loop less sensitive to nuclease. Moreover, there is a GT-AG rule proposed by Breathnach and Chambon, that nearly all introns of eukaryote genomic DNA coding proteins begin with GT and end with AG. In this case, the product of the direct amplification method contains two complete exon-intron boundaries. In spite of AT-cloning, this ihpRNA construct could be cloned into any vector at any clone site as long as primer

![Gene Map of Zm-abp1](image)

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3 got a 5′ heel of the corresponding enzyme digestion site, so that a special RNAi vector becomes less important. Using hpRNA constructs containing sense/anti-sense arms ranging from 98 to 853 bp gave efficient silencing in a wide range of plant species, and arms of 400–800 bp appear to be stable and effective.5 Therefore, the inverted arm should exceed 100 bp. The 3′ end of the intron-exon PCR product is reverse complementary to the 3′ end of the exon, and so the two parts can generate long inverted repeats on the ends and form stable panhandle-like structures after each denaturation-annealing PCR step.6 However, it appears that the resulting panhandle-like structure can hardly serve as a template for exponential PCR, because intramolecular annealing of longer sequences (beyond 100 bp) is both highly favored and more stable than intermolecular annealing of the much shorter PCR primers (about 20 bp).10 This is the suppressive PCR effect. Consequently, our recombinant-PCR mediated method can easily avoid this barrier to yield the right ihpRNA constructs.

In conclusion, this recombinant-PCR mediated ihpRNA construct generating method (RM-ihpRNA) is definitely capable of further improvement, allowing more precise predictions of gene function. It indicates a simple, reliable, and bright future for PTGS.

References


