**Short Communication**

**Simple RNAi Vectors for Stable and Transient Suppression of Gene Function in Rice**

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Since the recent sequencing of the rice genome, the functional identification of gene functions has become increasingly important. Various tagged lines have been generated; however, the number of tagged genes available is not sufficient for extensive study of gene function. To help identify the functions of genes in rice, we developed a Gateway vector, pANDA, for RNA interference of rice genes. This vector can be used for *Agrobacterium* transformation of rice and allows easy and fast construction of efficient RNAi vectors. In the construct, hairpin RNA derived from a given gene is transcribed from a strong maize ubiquitin promoter, and an intron is placed 5′ upstream of inverted repeats to enhance RNA expression. Analysis of rice genes using this vector showed that suppression of mRNA expression was observed in more than 90% of transgenic plants examined, and short interfering RNA indicative of RNA silencing was detected in each silenced plant. A similar vector, pANDA-mini, was also developed for direct transfer into leaf cells or protoplasts. This vector can be used for transient suppression of gene function in rice. These vectors should help identify the functions of rice genes whose tagged mutants are not available at present and complement existing methods for functional genomics of rice.

**Keywords:** dsRNA — Gene silencing — Rice — RNAi — Vector.

Abbreviations: RNAi, RNA interference; siRNA, short-interfering RNA; IR, inverted repeats.

RNA interference (RNAi) has been extensively used in various species to suppress gene function and is becoming a common tool for the functional analysis of the genome (Hannon 2002). Post-transcriptional gene silencing (PTGS) was first discovered in plants, and it is now believed that its molecular mechanism is similar to that of RNAi observed in *Caenorhabditis elegans*, *Drosophila*, and mammals (Matzke et al. 2001, Waterhouse et al. 2001). Two types of RNA play major roles in RNA silencing: dsRNA, which acts as a trigger of RNA breakdown, and short interfering (siRNA), which is involved in actual degradation of target mRNA in the final step of the RNAi pathway (Hannon 2002). Although various components of the RNAi pathway have been genetically and biochemically identified in several organisms, a complete picture of the RNAi pathway has not yet been revealed. More recently, microRNA has been discovered in various organisms including plants, and it has been shown to play important roles in development (Bartel and Bartel 2003). However, whether the pathways for RNAi and microRNA are overlapped or whether these pathways share any components remains to be studied.

Since it was shown for the first time that dsRNA can break down mRNA, which has complete homology with dsRNA (Waterhouse et al. 1998, Fire et al. 1998), this phenomenon called RNAi or RNA silencing has been extensively used to suppress gene function in plants. RNAi has been used for transient as well as stable suppression of gene functions in plants. Chuang and Meyerowitz (2000) first showed that dsRNA-mediated suppression of gene function was highly efficient in *Arabidopsis*. Transient suppression of gene function was also successfully demonstrated in various experimental systems in plants (Schweizer et al. 2000, Johansen and Carrington 2001). Wesley et al. (2001) developed generic vectors for the stable suppression of gene function by dsRNA, and they used a CaMV35S promoter for these vectors. In the same study, they showed a Gateway vector for high-throughput analysis of gene function by RNAi, which contains a 35S promoter and an intron in the linker region.

The rice genome was recently sequenced (Sasaki et al. 2002, Feng et al. 2002, Rice Chromosome 10 Sequencing Consortium 2003), and a number of gene-tagging methods have been developed or proposed to identify functions of numerous genes with unknown functions in rice (Hirochika 2001, Shimamoto and Kyozuka 2002). However, the predicted number of genes in rice was ca. 50,000, and existing plant lines, which are tagged by various kinds of tags, such as *Ac/Ds* transposons, *Tos17*, and T-DNA, are not yet sufficient to identify gene functions in rice. Furthermore, since hotspots for tagging are known in most of the tagging systems (Miyao et al. 2003) and various tagged lines are being grown in a number of different countries by various groups, it will be impossible to find ways to collect a large number of tagged lines for gene identification in the near future. These situations prompted us...
to develop vectors for high-throughput analysis of gene function based on the well-established RNAi method.

The destination vector pANDA that we developed contains the maize ubiquitin promoter with an intron (Christensen et al. 1992), which has been shown to give high expression of foreign genes in transgenic rice (Uchimiya et al. 1993, Shirasu et al. 1999, Hayama et al. 2003), and a 920 bp fragment of the coding region of the E. coli gus gene, which constituted a linker between two inverted repeats of the gene sequence derived from target genes (Fig. 1A). Since the 35S promoter is not as active in rice as it is in Arabidopsis or tobacco, and also to ensure high expression of hairpin RNA for gene suppression, we used the maize ubiquitin promoter and placed an intron upstream of inverted repeats (IRs) in the pANDA vector. The gus linker was used to examine the mRNA levels of hairpin RNA generated by the IR transgenes in rice plants.

To generate RNAi constructs for gene suppression, 300–500 bp fragments of gene sequences are generated by PCR from genes of interest, and the resulting PCR fragments are cloned into Gateway pENTR/D-TOPO cloning vector, which carry two recombination sites (attL1 and attL2) for LR Clonase reaction (Fig. 1B). Subsequently, the fragment derived from a target gene is transferred into a pANDA destination vector by recombinase reactions. In these reactions, the PCR-derived fragments are inserted into two regions flanked by two recombination sites (attB1 and attB2) in opposite directions, and the gus linker sequence is flanked by the two inverted repeats (Fig. 1). The pANDA vector is the binary vector for Agrobacterium-mediated transformation and has kanamycin and hygromycin resistance marker genes. The pANDA-mini vector is a derivative of pANDA and does not carry the sequences required for Agrobacterium transformation. It is used for direct transfer into leaf cells or protoplasts for transient suppression of gene function. Two boxes and a thin line downstream of pUbq are exons and an intron of the maize ubiquitin gene, respectively. (B) Vector construction using the pANDA or pANDA-mini vector. The sequence of a gene used for IR is amplified by PCR using primers. The forward primer should contain CACC at the 5' end for TOPO cloning. The PCR products are cloned into the pENTR/D-TOPO cloning vector (Invitrogen). The final RNAi vector was produced by an LR clonase reaction between the entry clone and pANDA or pANDA-mini vector.

Since phytoene desaturase gene (PDS) has been used to examine virus-induced gene silencing in both dicots and monocots because of its visible mutant phenotype caused by the lack of carotenoids (Liu et al. 2002, Holzberg et al. 2002), we tested the efficiency of RNA silencing for PDS expression by the pANDA vector. The 470 bp fragment of the rice PDS gene (Accession no. AF049356, nucleotide no. 1261–1730) was used to make IR, and the RNAi construct was introduced into rice by Agrobacterium-mediated transformation. Rice plants
transformed with the PDS-RNAi construct showed a clear albino phenotype (Fig. 2B–D), and the PDS mRNA levels were highly reduced (Fig. 2E). siRNA, a molecular marker for dsRNA-based gene silencing corresponding to the PDS sequence, was detected (Fig. 2F), confirming that the PDS-RNAi construct made using the pANDA vector efficiently suppressed PDS function in transgenic rice plants. Since two size classes of siRNA have been reported in plants showing RNA silencing (Hamilton et al. 2002, Papp et al. 2003), we examined PDS siRNA after purifying low-molecular-weight RNA. The results of the RNA analysis indicated that there were two size classes of siRNA: one class was approximately 25 nt, and the second was ca. 27 nt (Fig. 2G), confirming the presence of the two classes of siRNAs in rice (Kusaba et al. 2003). Since the Gateway system was used for construction of the RNAi vector, the final construct retained two 25 bp recombination sites (attB1 and attB2) flanking the IR sequence. Since these two extra sequences produce dsRNA in transgenic plants and potentially affect the efficiency of gene silencing, we made two RNAi vectors with essentially the same structure using the pANDA vector and a conventional construction method and compared the efficiency of gene silencing in transgenic rice plants. For this experiment, we employed the 3′UTR of OsRac4, which is a member of the OsRac small GTPase family (Kawasaki et al. 1999, Ono et al. 2001). OsRac4 is identical to OsRop4, which was described in a recently published paper (Christensen et al. 2003). The efficiency of gene silencing detected at the level of mRNA expression was virtually the same between the two constructs, and the effects of the extra sequences attaching the IR sequence were not observed (Fig. 3A). Furthermore, siRNA was similarly produced (Fig. 3B), and two distinct classes of siRNA for OsRac4/OsRop4 were also detected, confirming the results of PDS siRNA (Fig. 3C). The sizes of those siRNAs were ca. 23 nt and ca. 27 nt for OsRac4/OsRop4 (Fig. 3C). Although the molecular basis of the slight differences between the sizes of the siRNAs for PDS- and OsRac4/OsRop4-silenced plants remains to be studied it will be of interest to know whether two classes of rice siRNAs were functionally different, as shown in tobacco and Arabidopsis (Hamilton et al. 2002, Papp et al. 2003). Together, these results suggested that the extra sequences retained at both sides
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Simple RNAi Vectors for Stable and Transient

RNAi vectors for rice

We have tested the efficiency of gene suppression using a conventional vector whose structure was essentially identical to pANDA for 11 rice genes (Table 1). The results showed that, for all the 11 genes examined, gene suppression was observed in more than 80% of the transgenic plants examined. In almost all of the transgenic plants that failed to show silencing, RNAi constructs were not well transcribed, which was demonstrated by RT-PCR analysis of the gus mRNA derived from the linker region of the constructs. Therefore, when the RNAi constructs were expressed reasonably well, silencing was observed in almost all the transgenic plants.

Table 1 Efficiency of gene suppression by RNAi in rice

<table>
<thead>
<tr>
<th>Gene</th>
<th>No. silenced lines / No. obtained lines</th>
<th>Efficiency (%)</th>
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<tbody>
<tr>
<td>OsRac1</td>
<td>29 / 29</td>
<td>100</td>
</tr>
<tr>
<td>OsRac2</td>
<td>30 / 34</td>
<td>88</td>
</tr>
<tr>
<td>OsRac3</td>
<td>10 / 10</td>
<td>100</td>
</tr>
<tr>
<td>OsRac4/OsRop4</td>
<td>12 / 14</td>
<td>85</td>
</tr>
<tr>
<td>OsRac5/OsRacD</td>
<td>34 / 36</td>
<td>94</td>
</tr>
<tr>
<td>OsRac6/OsRacB</td>
<td>18 / 18</td>
<td>100</td>
</tr>
<tr>
<td>OsRac7/OsRop5</td>
<td>30 / 35</td>
<td>85</td>
</tr>
<tr>
<td>OsRac con1</td>
<td>86 / 88</td>
<td>97</td>
</tr>
<tr>
<td>OsRac con5</td>
<td>10 / 10</td>
<td>100</td>
</tr>
<tr>
<td>OsRad</td>
<td>10 / 11</td>
<td>90</td>
</tr>
<tr>
<td>PSD</td>
<td>25 / 27</td>
<td>92</td>
</tr>
</tbody>
</table>

Nomenclature of OsRac4-OsRac7 described in Christensen et al. (2003) is also shown. OsRac con1 and OsRac con5 are the sequences which are highly conserved among the members of OsRac gene family in OsRac1 and OsRac5, respectively. OsRad is a rice gene which has homology with yeast RAD2 gene and its function is unknown.

To test transient suppression of gene expression caused by direct introduction of the RNAi vector into rice cells, we generated the pANDA-mini vector, which carries a 300 bp fragment of the GFP gene. We co-bombarded the GFP RNAi construct, 35S-GFP, as a target gene and Ubq-DsRed as a control for bombardment efficiency. The results shown in Fig. 4A indicate that the bombarded 35S-GFP gave sufficient expression of GFP protein, which was evidenced by the green fluorescence in the rice sheath cells. When the GFP RNAi construct was co-bombarded with the 35S-GFP, strong silencing of the green fluorescence was observed (Fig. 4B), while no difference in the expression of the control DsRed gene was observed (Fig. 4C, D). These results suggested that the RNAi vector based on the pANDA-mini was useful for transient suppression of gene function in rice leaf cells.

Wesley et al. (2001) examined various RNAi vectors and found that those having an intron in the linker gave the most efficient suppression as reported by Smith et al. (2000). They tested the maize ubiquitin promoter in rice and showed that it gave high efficiency gene suppression. A Gateway vector, pHELLSGATE, which they developed for high-throughput RNA silencing, contains the 35S promoter. More recently the same group developed various derivatives of pHELLSGATE for efficient construction and gene suppression (Hilliwell and Waterhouse 2003). Similar intron-containing Gateway vectors driven by the 35S promoter have been described (Karimi et al. 2002). For transient suppression of gene function in cereals 35S-based (Schweizer et al. 2000) and pUbq-based RNAi vectors (Azvvedo et al. 2002) have been used for bombardment of leaf cells. Panstruga et al. (2003) developed an assay to test the efficiency of dsRNAi constructs by the use of two fluorescent
proteins: one used as a marker and the second as translational fusion with the target gene. Therefore, a variety of RNAi vectors including pANDA are now available for suppression of gene function in plants. They should be useful tools for the identification of gene function in plants.

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Fig. 4 Transient suppression of GFP gene expression in rice leaf cells by pANDA-mini-based vector. (A, B, G, H) GFP fluorescence. (C, D, I, J) DsRed fluorescence. (E, F, K, L) Merged images. (A) (C) (E) (G) (I) (K) Control experiment. Leaf sheath cells were co-bombarded with p35S-GFP, pUbq-DsRed, and the empty pANDA-mini vector. (B, D, F, H, J, L) Leaf sheath cells were co-bombarded with p35S-GFP, pUbq-DsRed, and GFP RNAi vector using the pANDA-mini vector. (G–L) The synthetic oligonucleotide primers: forward 5'-CACCGGCTGCGTAATATCCAGCC-3', reverse 5'-TGTTGGGTCTATATCTGAAGTTACC-3' were used for the amplification of the IR region for the GFP gene. A transient expression assay was performed by particle bombardment of leaf sheath cells of young rice plants by the use of the PDS-1000/He system (Bio-Rad Laboratories). For the co-bombardment assay, 2 µg each of p35S-GFP and pUbq-DsRed plasmids and 6 µg of the GFP RNAi vector or empty pANDA-mini vector were introduced into leaf sheath cells of cv. Kinmaze. The fluorescence of the GFP and DsRed proteins was observed with a fluorescence stereo-microscope (MZ FL III, Leica Microsystems) following incubation for 48 h at 30°C.

References


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