Plant Cell Physiol. 45(4): 490–495 (2004)
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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 rice genes 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

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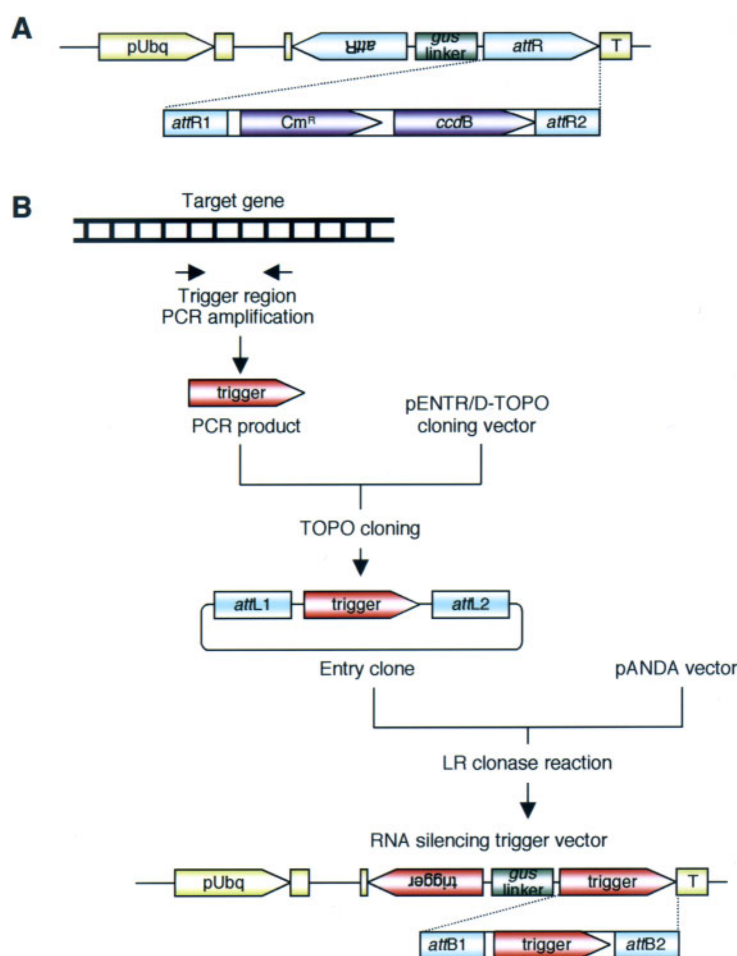

Fig. 1 Structure of the pANDA vector and procedure for RNAi vector construction. (A) Diagram of part of the pANDA and pANDA-mini vectors containing the promoter and IR regions. The Gateway rf A cassette (Gateway vector conversion system; cat. No. 11828–019, Invitrogen) was cloned into both sides of the *gus* linker (920 bp) region in the antisense and sense orientation by insertion into the *Eco*RV and *Spe*I–*Sac*I restriction enzyme sites of pGUS27. The inserted IR sequences and the *gus* linker were cloned into the *Kpn*I–*Sac*I restriction enzyme sites of a p2K-1+ vector that carried the maize ubiquitin (*Ubq*) promoter and an intron. The LR clonase reaction site *att*R is located at both sides of the *gus* linker in the antisense and sense orientations. 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.

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. 1B). The pANDA vector was developed for *Agrobacterium*-mediated transformation and carried the kanamycin- and hygromycin-resistance markers for plant transformation. The pANDA-mini vector was designed for direct transfer of the gene sequence into rice cells by particle bombardment or electroporation of rice protoplasts. The pANDA-mini vector is essentially the same as the pANDA vector except that it contains no sequences required for *Agrobacterium* transformation.

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

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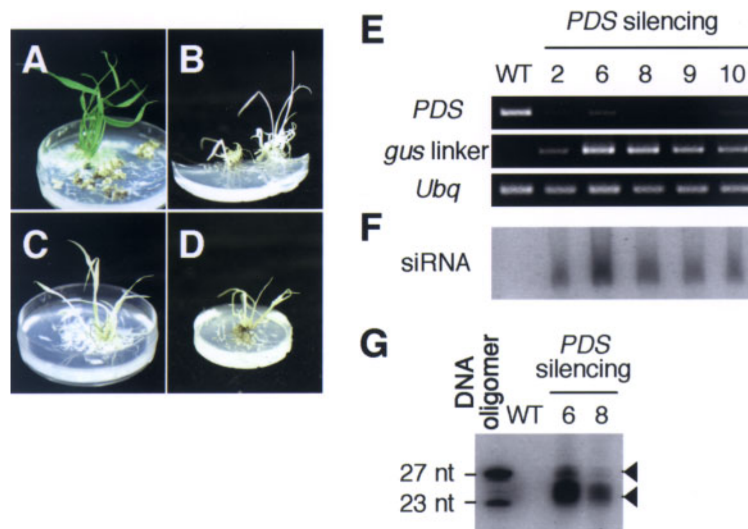

Fig. 2 Silencing of rice phytoene desaturase (PDS) gene expression by the pANDA-derived vector. (A) Control plant. (B–D) Three independent *PDS* silencing transgenic rice plants showing the albino phenotype. (E) RT-PCR analysis of *PDS* RNAi plants. Five independent transgenic rice plants (2, 6, 8, 9, 10) showed reduced *PDS* mRNA accumulation. The *gus* linker indicates RT-PCR products of the *gus* linker region, indicative of the expression of the trigger dsRNA. *Ubq*, rice ubiquitin gene used as a control. (F) Detection of *PDS* siRNA. The siRNA was detected in the same transgenic rice plants shown in (E). (G) Two classes of *PDS* siRNA. The siRNA detected in (F) was further purified and subjected to hybridization. The synthetic oligonucleotide primers: forward 5′-CACCTTATGCGGACATGTCAGTAACTT-3′, reverse 5′-CCATTGGGAATAGTCCTGACTAC-3′ were used for the amplification of the IR region for the *PDS* gene. The short and long siRNAs (arrowheads) were detected in two independent transgenic rice plants. Twenty-three and 27 nt are the sizes of the DNA oligomers used as markers. Transgenic rice plants were produced by *Agrobacterium*-mediated transformation of rice calli (cv. Kinmaze) according to a published protocol (Hiei et al. 1994). Total RNA was isolated according to a published method (Chomczynski and Sacchi 1987). For the synthesis of cDNA, 1 µg of total RNA was primed using oligo d(T) primers according to standard procedures (Super Script II, Invitrogen). In each experiment, 50 ng cDNA was used for PCR amplification with each of the following gene-specific primer sets: *PDS*, 5′-TGCAATGGAAGGAACACTCC-3′ and 5′-TACGAGAATTCAGCCGAACC-3′; *gus* linker, 5′-CATGAAGATGCGGACTTACG-3′ and 5′-ATCCACGCCGTATTCGG-3′; and *Ubq*, 5′-CCAGGACAAGATGATCTGCC-3′ and 5′-AAGAAGCTGAAGCATCCAGC-3′. siRNA was detected and separated according to a published protocol (Hamilton and Baulcombe 1999). To purify RNA for separation of two size classes of siRNA, a protocol for isolation of low-molecular-weight RNA (QIAGEN) was used with the RNA/DNA System (QIAGEN).

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 *PDS* 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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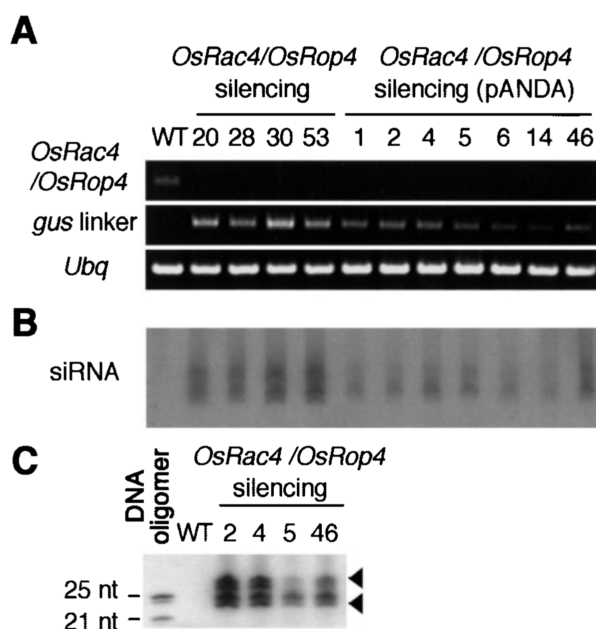

Fig. 3 Comparison of the efficiency of gene silencing by the pANDA-based vector and a similar construct made using the conventional method. (A) RT-PCR analysis of transgenic rice plants. Four independent transgenic rice plants (20, 28, 30, 53), which were silenced by the construct made by restriction enzymes, and seven independent transgenic rice plants (1, 2, 4, 5, 6, 14, 46), which were silenced by the pANDA-based vector, were examined for *OsRac4/OsRop4* mRNA expression. The *gus* linker indicates the RT-PCR products of the *gus* linker region, indicative of the expression of the trigger dsRNA. *Ubq*: rice ubiquitin gene used as a control. (B) Detection of *OsRac4/OsRop4* siRNA. The siRNA was detected in the same transgenic rice plants shown in (A). (G) Two classes of *OsRac4/OsRop4* siRNA. The siRNA detected in (B) was further purified and subjected to hybridization. The synthetic oligonucleotide primers: forward 5′-CACCAGAAGAAGGCTAGTATGCTTCA-3′, reverse 5′-ATGTAGGGAGTGCTAGGAACCTT-3′ were used for the amplification of the IR region for the *OsRac4/OsRop4* gene. The short and long siRNAs (arrowheads) were detected in four independent transgenic rice plants. Twenty-one and 25 nt are the sizes of the DNA oligomers used as markers. Transgenic rice plants were produced by *Agrobacterium*-mediated transformation of rice calli (cv. Kinmaze) according to a published protocol (Hiei et al. 1994). The methods used to detect *OsRac4/OsRop4* mRNA were the same as those described in Fig. 2. The primers used for RT-PCR analysis were 5′-GGGACCAAATTGGATCTTCGTG-3′ and 5′-CCATTCTGAGATGTAGGGAGTGC-3′.

of the IR sequence in the pANDA-based RNAi vector do not affect the silencing efficiency.

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

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

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 (Helliwell 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 (Azevedo 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

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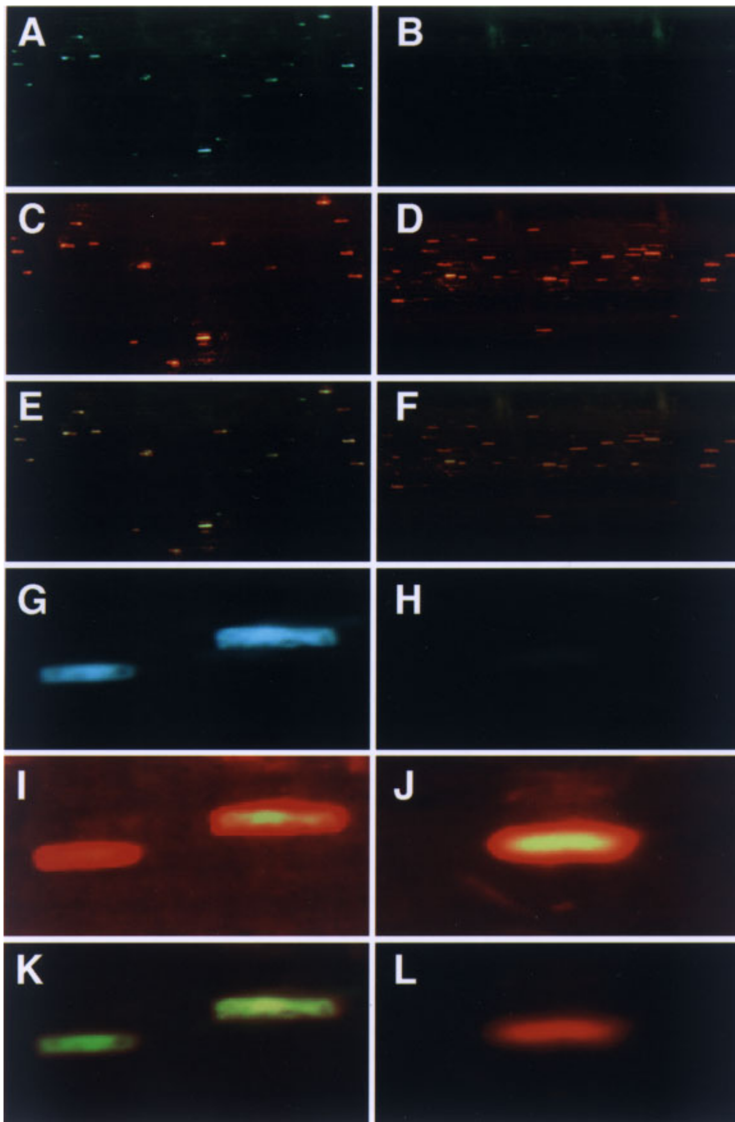

Fig. 4 Transient suppression of GFP gene expression in rice leaf cells by pANDA-mini-based vector. (A, B, G, H) GFP fluorescence. (C, D, I, J) DsRed fluorescence. (E, F, K, L) Merged images. (A) (C) (E) (G) (I) (K) Control experiment. Leaf sheath cells were co-bombarded with p35S-GFP, pUbq-DsRed, and 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′-CACCGGCGTGCAGTGCTTC-AGCC-3′, reverse 5′-TGTTGTGGCTTATCTTGAAGTTC-ACC-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.

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.

Acknowledgments

We thank Dr. Junko Kyozuka at Tokyo University for pGUS27 and p2K-1^{+}. We thank members of the Plant Molecular Genetics Lab at NAIST for their comments and participation in discussions. This work was supported by the Research for the Future Program of the Japan Society for the Promotion of Science (grant no. JSPS-RFTF00L01604).

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(Received December 3, 2003; Accepted January 27, 2004)

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