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Minireview

Dissection of gene function by RNA silencing

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Abstract The RNA silencing technique is an effective tool to examine the biological function of the target mRNA in plants. The recent development of versatile-type RNAi vectors, which are driven by constitutive promoters, and GATEWAY™ cloning technology makes it easy to construct the RNAi vectors with trigger sequences and to analyze the function of a target gene. Although these vectors are highly useful, constitutive defects of the target mRNA expression sometimes result in lethality or seed abortion. Here, we summarize recent approaches to RNA silencing research designed to overcome these difficulties and to dissect gene expression.

Key words: RNA silencing, RNAi vector, *Waxy* gene.

RNA interference (RNAi) is a process of post-transcriptional mRNA degradation that is triggered by double-stranded RNA (dsRNA) or 21–26 nt small interfering RNA (siRNA) (Pickford and Cogoni 2003; Vance and Voucheret 2001; Waterhouse et al. 2001; Hannon 2002). The latest achievement in RNA technology, RNA-mediated gene silencing, was demonstrated in model eukaryotic organisms (Fire et al. 1998; Waterhouse et al. 1998; Misquitta and Paterson 1999; Elbashir et al. 2001; Jacque et al. 2003; Rapoli and Arndt 2003; Schramke and Allshire 2003). In plant, stable transformation with a vector can form dsRNA is generally used for RNA-mediated gene silencing system, and siRNA is commonly observed in these transgenic plants as RNAi (Wesley et al. 2001; Klahre et al. 2002). Recent studies have shown that inducible and tissue-specific RNAi vectors have been developed and are available for high-throughput analyses for functional genomics.

Construction of RNAi vectors is facilitated by the GATEWAY™ cloning system

GATEWAY™ technology (Invitrogen, Carlsbad, CA; URL: http://www.invitrogen.com./content/sfs/manuals/gatewayman.pdf) is based on the site-specific recombination reaction of λ phage. The technology removes the burden of vector construction by using conventional cloning techniques, and PCR-amplified trigger sequences can be easily cloned into RNAi vectors as directed orientations.

Smith et al. (2000) reported that the intron containing a self-complementary RNA vector was the most efficient RNAi vector and the spliceable intron could enhance silencing efficiency. Later, improved vectors were developed with a commonly used constitutive CaMV35S promoter. The vectors can be easily cloned through an inverted repeat sequence homologous to the target gene by using GATEWAY™ technology with the intron-spacers of various genes or different orientations of the intron-spacers (Wesley et al. 2001; Karimi et al. 2002; Helliwell and Waterhouse 2003; Helliwell and Waterhouse 2005). A similar GATEWAY™ RNAi vector carrying a maize *ubiquitin1* promoter and *gusA* linker was developed (Miki and Shimamoto 2004) and was shown to be effective for both gene-specific disruption and the disruption of multiple members of family genes by using solely or tandemly connected gene-specific sequences or solely conserved sequences for analyses of the rice phytoene desaturase (PDS) gene and the small GTPase Rac1 gene family (Miki et al. 2005). These vectors were highly effective in suppressing target mRNA expression, and the efficacy of suppression varied from 85 to approximately 100% among transgenic lines for 11 target genes.

Abbreviations: dsRNA, double-strand RNA; GA, gibberellin; GBSS, granule-bound starch synthase; GFP, green fluorescence protein; ihpRNA, intron-spliced hairpin RNA; hpRNA, hairpin RNA; PDS, phytoene desaturase; RNAi, RNA interference; *Wx*, *Waxy* gene; siRNA, small interfering RNA.

This article can be found at http://www.jspcmb.jp/

Chemical- and stress-inducible RNAi vectors

RNAi vectors were driven by constitutive or strongly active promoters, leading to defects in the target mRNA expression. When the target mRNA is essential for the viability of the cells, complete suppression throughout development leads to lethality, seed abortion, or loss of plant regeneration, in some cases making studies very difficult. To control temporal and spatial suppression of target mRNA expression, chemical or abiotic, environmental stress-inducible RNAi vectors have been proposed.

17β-estradiol-inducible, chemical-inducible RNAi vectors were developed based on a P1 phage *Cre*/*loxP* recombination system by Guo et al. (2003). The chemical-inducible RNAi vector can be used to produce ihpRNA by β-estradiol treatment as follows. β-estradiol activates XVE via a chimeric transactivator containing the estrogen receptor regulator domain. Then XVE activates Cre by binding to the *lexA*/-46CaMV35S promoter located upstream of *Cre*. After the Cre protein is expressed, *XVE*, *Hm*^{r} and *Cre* genes intervening between the *loxP* sequences are excised from the original constitution at an integrated site by homologous recombination. After the recombination, G10-90, a distal and strong constitutive promoter, fuses to an inverted repeat sequence homologous to the target gene and enables the transcription of the functional ihpRNA. They tested the effect of the β-estradiol-inducible vector on suppression of *GFP* and *PDS*. The inverted repeat of the partial *GFP* or *PDS* sequences into the cloning site of the vector, and resultant vectors effectively suppressed the expression of both target genes, *GFP* and *PDF*, by β-estradiol treatment in the transgenic *Arabidopsis* (Guo et al. 2003). The efficacy of the transgenic lines showing a loss of green fluorescence and photobleaching were almost 100% of the plant tested. All the β-estradiol-inducible *PDS* RNAi plants were fertile and overcame lethality, whereas most control CaMV35S::PDSRNAi plants showed abnormal development and poor fertility. Furthermore, almost progeny from β-estradiol-induced silencing lines showed a reversed phenotype in the absence of the inducer, and inducer-dependent suppression was shown even in the progeny from the lines showing an irreversibly silenced phenotype. Advantages of this system are not only effective and inducible RNAi but also the availability of selective marker-free disruptants of the target gene.

Another chemical-inducible RNAi vector was reported by Chen et al. (2003), who constructed an alcohol-inducible RNAi vector by using a CaMV35S-AlcR transcriptional regulator and an *alcA* promoter-regulated inverted repeat sequence homologous to the target gene. In the presence of ethanol, AlcR binds to the modified *alcA* promoter and activates the transcription of inverted repeat sequence. Because the target genes, magnesium-chelatase subunit 1 (*ChlI*) and glutamate 1-semialdehyde aminotransferase (*GSA*), are involved in chlorophyll biosynthesis, transgenic tobacco lines carrying the alcohol-inducible RNAi vectors targeting either *ChlI* or *GSA* mRNA showed visibly silenced phenotypes with a white, yellow or pale green color on the leaves after alcohol treatment in a short period of time. The silencing was not appeared in newly developed leaves and was available for temporal and spatial expression of the target genes. However, the silencing efficiency of the alcohol-inducible RNAi system was lower than that of the β-estradiol-inducible RNAi system. In the case of GSA disruption by alcohol-inducible RNAi, only 13% of the obtained plants were silenced.

A simpler system for inducible RNAi, a heat-shock promoter driving an ihpRNA vector, was reported by Masclaux et al. (2004). The *HSP18.2* heat-shock promoter, driving the ihpRNA gene homologous to *PDS*, led to the photobleaching in transgenic *Arabidopsis* and suppressed *PDS* mRNA expression at 37°C. Common features of these three types of the inducible RNAi vectors are escape lethality and the reversibility of the silencing state at the newly formed organ or next generation in the absence of the inducers.

Tissue-specific RNAi vectors

A tissue-specific promoter is available for the dissection of pleiotropic expression and the analysis of spatial disruption in viable transgenic plants. In *Arabidopsis* floral formation, the combined functions of the A-type, B-type and E-type MADS box genes lead to the formation of petals. A recent paper showed that the A-type MADS box gene *APETALA1* (*AP1*) promoter regulated the expression of ihp RNA genes homologous to the respective B-type MADS box genes *APETALA3* and *PISTILATA* (*AP3*, *PI*), showing the suppression of petal formation and the emergence of double sepals in flowers of transgenic *Arabidopsis* (Byzova et al. 2004). Therefore, a combination of organ-specific target gene expression and another organ-specific promoter regulating RNAi enables a more confined spatial disruption of the target mRNA expression and is useful for the dissection of gene function for organ formation.

Tissue- or organ-specific RNAi is useful not only for gene function research but also for the improvement of plant-derived products. Stautjesdijk et al. (2002) reported that ihpRNA-mediated RNAi targeted to the *Arabidopsis FAD2* Δ12 desaturase gene showed suppressed expression of *FAD2* among all transgenic *Arabidopsis* under the regulation of the seed-specific promoter of *napin* from oilseed rape. The resulting transgenic *Arabidopsis* showed changes in the fatty acid

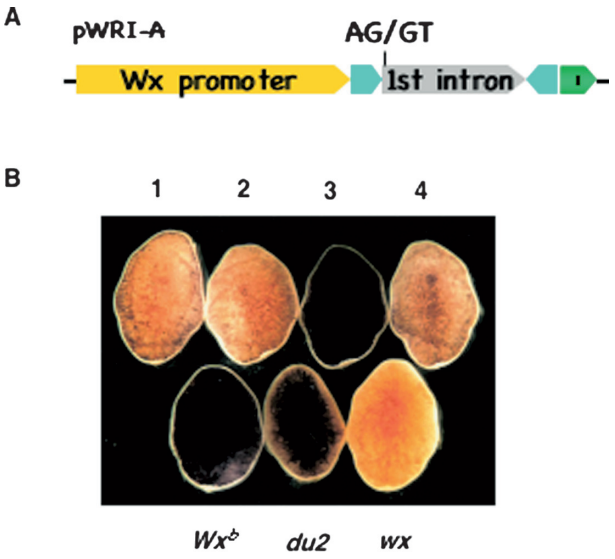

Figure 1. pWRI-A strongly suppressed *Wx* gene expression in rice seeds. The *Wx* gene encodes Granule-Bound Starch Synthase (GBSS) and plays a key role in amylose synthesis in rice. The *Wx* promoter regulates endosperm-specific *Wx* transcription in rice seeds. The *Wx* promoter fused to the *Wx* 5′UTR inverted repeat gene with the Wx^a 1st intron spacer, a resultant pWRI-A strongly suppressed *Wx* expression in transgenic rice seeds. A) Structure of pWRI-A. Blue-colored boxes show 5′UTR sequences. The 1st intron is derived from the Wx^a gene (Isshiki et al. 1998). B) R1 seeds from a rice plant were transformed with pWRI-A, and the mature seeds were sliced and stained with potassium iodide. Upper row: transgenic R1 seeds. Lower row: Wx^b, *Oryza sativa* L. cv. Kimmaze; *du2*, low amylose mutant; *wx*, cv. Musashimochi.

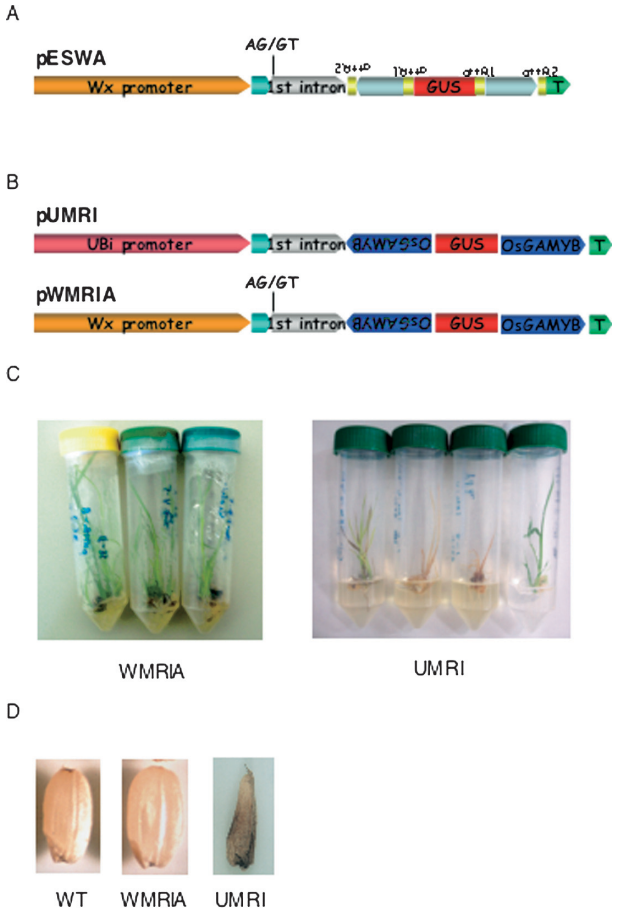

Figure 2. Analysis of the *OsGAMYB* gene by using an endosperm-specific RNAi vector, pESWA. A) Structure of pESWA. B) Structures of pUMRI and pWMRIA. Both vectors carry the same *OsGAMYB* sequence as a trigger sequence. The same PCR-amplified fragments of the partial OsGAMYB were cloned into pANDA (Miki and Shimamoto 2004) and pESWA, and the resulting vectors were named pUMRI and pWMRIA, respectively. C) Transgenic rice plants carrying pWMRIA (left) and pUMRI (right). D) Transgenic rice seeds carrying pWMRIA (center) and pUMRI (right).

composition, the fatty acid composition ratio and the oleic acid desaturation proportion of the most strongly silenced lines equivalent to those of the *fad2-1* mutant. Davuluri et al. (2005) also reported that fruit-specific regulated RNAi led to the disruption of the expression of the photomorphogenesis regulatory gene, *DET1*, in tomato and resulted in an increase in carotenoid and flavonoid contents.

We have demonstrated that the seed-specific promoter *Wx* fused to the *Wx* ihpRNA gene homologous to 5′UTR of *Wx* mRNA showed strong suppression of *Wx* mRNA and resulted in the loss of amylose synthesis in rice seeds (Figure 1) (Tanno et al. 2005). Furthermore, we have developed the seed-specific RNAi vector, pESWA, and we have shown that the PCR-amplified trigger sequences can be easily cloned into pESWA as an inverted repeat orientation by the GATEWAY™ system (Figure 2A). pESWA can be used for the analysis of genes functioning in the endosperm; e.g., genes involved in starch biosynthesis are possible targets of endosperm-specific RNA silencing by pESWA. As another target, genes showing pleiotropism can be dissected by endosperm-specific RNAi. To demonstrate the latter case, we tested pESWA in an analysis of the *GAMYB* gene in rice. The *OsGAMYB* gene shows pleiotropic expression. The loss of *OsGAMYB* function leads to strong lethality in pollen and seed (Kaneko et al. 2004) and blocks plant regeneration (Figure 2C, right). We cloned the *OsGAMYB*-inverted repeat sequences into both the RNAi vector with a constitutive promoter, pANDA (Miki and Shimamoto 2004), and the endosperm-specific RNAi vector, pESWA. The resulting vectors were named pUMRI and pWMRIA, respectively (Figure 2B). Transgenic R0 UMRI plants showed slow growth and often lethality (Figure 2C, right), whereas the transgenic R0 WMRIA plants showed normal growth (Figure 2C, left) at 2 weeks after regeneration. Furthermore, all the UMRI lines showed complete seed abortion and incomplete embryo development, whereas all the WMRIA lines showed fertility, and the seeds were slightly lager than those of WT (Figure 2D). These results suggest that *OsGAMYB* has an essential function in endosperm formation through embryo development at the early stage of seed development in rice. Endosperm-

specific RNAi revealed the *OsGAMYB* function in the seed development of rice.

Concluding Remarks

Few investigations have been carried out regarding RNAi vectors for spatial and temporal dissection of target gene function. In the next few years, a combination of an inducible promoter or a tissue-specific promoter, used with the GATEWAY™ cloning system, will be major structures of RNAi vectors for the dissection of gene function in plant research. Further improvement or development of these inducible, tissue or developmental stage-specific RNAi vectors will accelerate the progress of functional genomics.

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