# Intron Loop *Wx* 5'UTR dsRNA Vectors Mediated Endosperm Specific Silencing of *Wx* Gene of Rice

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**Summary**

To evoke endosperm specific disruption of gene function and quantitatively regulated RNAi, ihp dsRNA vectors, pWRI-A and pWRI-B, were constructed by using endosperm specific promoter and 1st intron of *Wx* gene. pWRI-B carries a single mutation at the 5' splicing site of 1st intron and the mutation reduces the mature transcript of the dsRNA gene. Both vectors efficiently disrupted *Wx* mRNA function among 96-100 % of transgenic rice, and the degrees of *Wx* gene suppression at *WRI-B/Wx*$^b$ endosperms were weaker than that of *WRI-A/Wx*$^b$ endosperms. These results clearly showed the *Wx* promoter regulates endosperm specific RNAi. Moreover, the controlling the splicing efficiency can quantitatively regulates the suppression effect on RNAi.

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Abbreviations: RNAi, RNA interference; *Wx*, *Waxy* gene; GBSS, Granule-bound starch synthase; NMD, nonsense-mediated mRNA decay; siRNA, small interfering RNA

**Key words:** RNA interference, splicing efficiency, *Oryza sativa* L., Granule-bound starch synthase, *Waxy*, siRNA

RNA interference (RNAi) is a process of post-transcriptional mRNA degradation that is triggered by double-stranded RNA (dsRNA) (Pickford and Cogoni 2003, Vance and Voucheret 2001, Waterhouse et al. 2001, Hannon 2002). As latest achievement of RNA technology, RNA-mediated gene silencing demonstrated in many eukaryotes, *Caenorhabditis elegans* (Fire et al. 1998), plants (Waterhouse et al. 1998), *Drosophila* (Misquitta and Paterson 1999), mamalian cell (Elbashir et al. 2001, Jacque et al. 2003), and in fission yeast (Rapoli and Arndt 2003, Schramke and Allshire 2003).

In plant, stable transformation with vector can form dsRNA is generally used for RNA-mediated gene silencing system, and small interfering RNA (siRNA) is commonly observed in these transgenic plants as like RNAi (Wesley et al. 2001, Klahre et al. 2002). Therefore, the RNA-mediated gene silencing by stable transformation system with vector can form dsRNA in plant is now referred to as RNAi.

Recent studies showed that the various RNAi vectors were developed and available for high-throughput analyses for functinal genomics. The chemical inducible RNAi vector was developed in order to avoid the embryonic lethality (Guo et al. 2003, Chen et al. 2003). High throughput RNAi vectors with inverted repeat of a heterologous 3' untranslated region was developed to easy construction for gene disruption (Brummell et al. 2003). Furthermore, intron containing self-complementary RNA vector was reported as a most efficient RNAi vector and the spliceable intron could enhance the silencing efficiency (Smith et al. 2000, Wesley et al. 2001, Miki and Shimamoto 2004). But the GU-AG intron effect on RNAi has not been evaluated yet adequately, as the authors pointed out the possibility that the spacer intron act as the transcription enhancer (Wesley et al. 2001). If the splicing system of the GU-AG intron can affect the efficiency of RNAi, the mutation of the intron consensus sequence may disrupt or reduce the silencing efficiency.

The *Waxy* locus encodes granule-bound starch synthase (GBSS), which is a key enzyme in amylose synthesis in rice. The *Wx* gene is specifically expressed in pollen, and endosperm and the gene presents as a single copy gene in rice genome. In rice, two functional alleles, *Wx*$^a$ and *Wx*$^b$ (Sano 1984, Sano et al. 1986), are exist. *Wx*$^b$ carries a +1G to T mutation at the 5' splice site of the first intron (Isshiki et al. 1998, Hirano et al. 1998), and the mutation caused lower levels of *Wx* mRNA accumulation in developing endosperm. Previous investigations for antisense suppression, co-suppression, and disruption by gene targeting in rice *Wx* gene had been reported (Itoh et al. 1997, Terada et al. 2000, Terada et al. 2002). However, stable and efficient suppression of *Wx* and quantitative regulation of *Wx* expression are still technological problems should be solved.

In this study, we constructed endosperm specific ihp-dsRNA vectors targeted to 5'UTR *Wx* mRNA, pWRI-A and pWRI-B. pWRI-B carries the mutant sequence at 5' splice site of 1st intron, whereas pWRI-A carries wild type. These vectors were transformed into *Wx*$^b$ rice, and resulting transgenic plants *WRI-A/Wx*$^b$ and *WRI-B/Wx*$^b$ were tested and discussed for spatial and quantitative control of silencing

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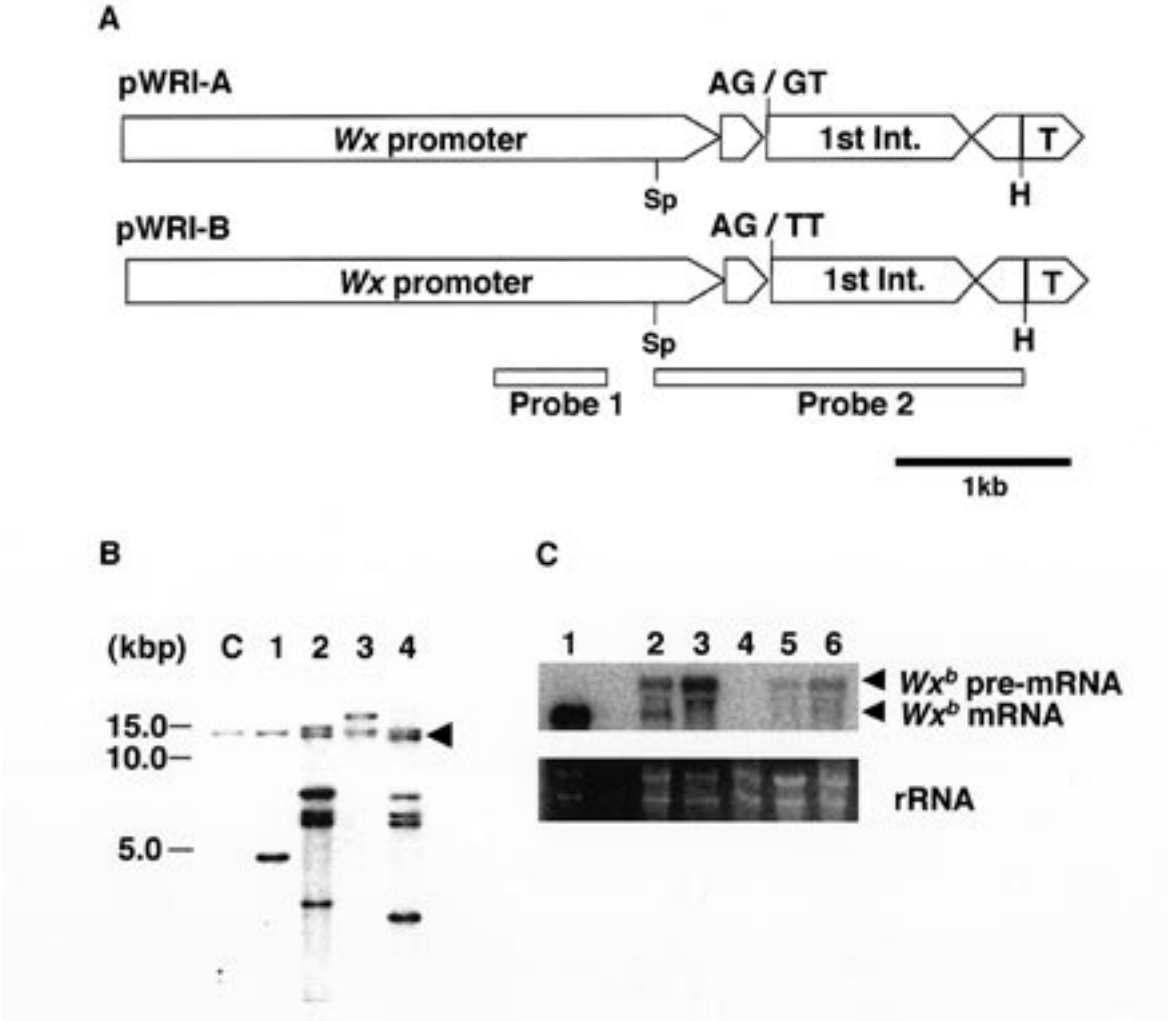


**Fig. 1.** Structures, integration, and silencing effects of the *WRI* genes. **A**, Structures of *WRI-A* and *WRI-B*. Both two genes have *Wx* promoter, sense and antisense directions of *Wx* 5'UTR region, *Wx* first intron, and a terminator of the CaMV-35S. The consensus sequences of GU-AG intron is wild-type at *WRI-A*, whereas the *WRI-B* has G to T mutation at the 5' splice site of the first intron derived from $Wx^b$ gene. *Arrows* indicate the sense or antisense direction of 0.14 kbp 5'UTR (1st exon) of *Wx* gene. The *open boxes* show the sequences that were used for the hybridization probes. The Probe 1 was used for Southern-blot analysis (panel B) and the Probe 2 was used for the small RNA detection (see Fig.4). *Abbreviations*: Sp, restriction site of *Sph* I. H, restriction site of *Hin*dIII. **B**, Southern blot analysis of $WRI/Wx^b$ transgenic lines. Genomic DNAs were isolated from mature leaves and were digested with *Hin*dIII. Southern hybridization was performed with the Probe 1 (see panel A). Lane C, *Oryza sativa* L. cv. Kinmaze; lane 1-2, two independent $WRI\text{-}A/Wx^b$ transgenic R0 lines, line WRI-A18, line WRI-A42, respectively; lane 3-4, two independent $WRI\text{-}B/Wx^b$ transgenic R0 lines, line WRI-B4, line WRI-B39, respectively, *Arrowhead* indicates signal of endogeneous $Wx^b$ (ca. 14.7kb). **C**, degradation of the $Wx^b$ mRNA at $WRI/Wx^b$ transgenic rice. Total RNAs were isolated from developing seeds at 14 days after pollination and the 2 μg of the total RNA was loaded on each lane. Lane 1, *Oryza sativa* L. cv. Labelle ($Wx^a$); lane 2, cv.Kinmaze ($Wx^b$); lane3, cv. Musashimochi ($wx^{mmd}$); lane4, 75KURwx4 (deletion mutant, $wx^{del}$); lane 5, $WRI\text{-}A/Wx^b$ plant carrying with a single copy of *WRI-A* gene (line WRI-A18); lane 6, $WRI\text{-}B/Wx^b$ plant carrying with a single copy of *WRI-B* gene (line WRI-B4).

efficiency.

## Materials and Methods

### *Plant materials*

All seeds were sown and cultivated at 26～28°C in a greenhouse under both natural light and supplemental light with long-day condition (14 hr light/ 10 hr dark). *Oryza sativa* L. Japonica cv. Kinmaze ($Wx^b$), *O. sativa* L. Indica cv. Labelle ($Wx^a$), *O. sativa* L. Japonica cv. Musashimochi (*wx*), line EM2 (*du-2*) and line 75KURwx4 (*wx*) were used as controls. Cv. Kinmaze was also used for genetic transformation. We

named the two *wx* alleles $wx^{mmd}$ (cv. Musashimochi) and $wx^{del}$ (75KURwx4) as tentative gene nomenclatures in this manuscript. Mutant line EM2 was provided by Professor H. Sato, Kyushu University and line 75KURwx4 was provided by Professor Y. Amano, Fukui Prefectural University.

***Plasmid constructs***

*WRI-A* and *WRI-B* genes have same structure except a single substitution mutation at the *Wx* 1st intron. Both genes were constructed from 2.7 kbp fragment of the *Wx* promoter, 0.14 kbp sense direction of *Wx* 1st exon, 1.1 kbp of *Wx* 1st intron, 0.14 kbp antisense direction of *Wx* 1st exon, and 0.2 kbp CaMV-35S terminator (Fig. 1A). A major difference between *WRI-A* and *WRI-B* is a +1G to T mutation of the splicing consensus sequence at the 5' splice site of the first intron, and the mutation may result reducing of mature transcripts (Isshiki et al. 1998). The resulting transcripts of both genes will be intron-spliced double-stranded RNA homologous to 1st exon of *Wx* mRNA (Fig. 1B).

Each gene, *WRI-A* and *WRI-B*, was inserted into multiple cloning site of pPZP2H-lac (Fuse et al. 2001), and resultant pWRI-A and pWRI-B were transformed into *Agrobacterium tumefaciens* strain EHA101, respectively. Plasmid pPZ2H-lac was provided by Dr. Yano, National Institute of Agrobiological Sciences, Tsukuba, Japan.

***Rice transformation***

Rice calli were initiated from mature dry seeds of cv. Kinmaze ($Wx^b$) and were transformed by *Agrobacterium*-mediated gene transfer (Holster et al. 1978, Hiei et al. 1994, Toki 1997, Terada et al. 2002). The copy numbers of the transgene per diploid of all R0 transgenic lines were determined by Southern blot analysis and PCR analysis of the transgenes.

***Phenotypic assay***

To examine the silenced *Wx* expression in *WRI*/$Wx^b$ transgenic plants, mature R0 pollen and R1 seeds were collected and then stained by potassium iodide.

***Southern blot analysis***

Genomic DNAs were isolated from young leaves of transgenic plants (Walbot and Warren, 1988) and 5 $\mu$g DNAs were digested with *Hin*dIII, then electrophoresed in a 0.7 % agarose gel, and then transferred onto a positively charged nylon membrane (Roche diagnostics K.K.). A 0.5 kbp fragment of *Wx*-promoter region (Fig. 1A Probe1) was amplified by PCR and used for a hybridization probe was labeled with [$\alpha$-$^{32}$P]dCTP using the *Redi*-prime labeling system (Amersherm Biosciences K.K.). Hybridization and washing procedures were performed according to the manufacturer's protocol.

***Northern blot analysis***

Total RNA was isolated from developing endosperm at 14 days after pollination according to a published method (Nagy et al. 1988). 2 $\mu$g of total RNA was separated by electrophoresis in 1.2% agarose gels containing formaldehyde and then transferred onto the positively charged nylon membrane (Roche Diagnostics K.K). Hybridization was performed with antisense RNA probe (Fig. 1A Probe 2) that had been labeled with [$\alpha$-$^{32}$P]UTP using Riboprobe *in vitro* Transcription Systems (Promega Co.).

***siRNAs detection***

200 $\mu$g of total RNA was extracted from developing endosperms at 14 days after pollination, and then the low molecular weight RNA spieces were purified by a polyethylene glycol precipitation (Sambrook et al. 1989). The RNAs were separated in 12% polyacrylamide gel by electrophoresis, and then blotted onto the nylon membrane, Hybond NX (Amersherm Biosciences K.K.). Detection of the small interfering RNA (siRNA) was performed according to Hamilton and Baulcombe (1999).

***Signal detection***

The hybridization signals were detected by contacting with the Imaging Plate (Fuji Photo Film Co. Ltd.), and then the signal intensity was measured by BAS-5000 system (Fuji Photo Film Co. Ltd.).

## *Results*

To evoke the endosperm specific silencing and the quantitatively regulated RNAi, two ihp RNAi vectors, pWRI-A and pWRI-B, were constructed (Fig. 1A) and transformed into non-*waxy* rice ($Wx^b$). Both pWRI-A and pWRI-B have almost identical structure for targeting to 5'UTR of *Wx* mRNA, and essential component of *WRI* genes are the *Wx* promoter, 5' UTR sense and antisense sequences, *Wx* 1st intron as a spacer sequence, and 35S terminator. The pWRI-B has a substitution mutation at the 5' splice site of the *Wx* 1st intron, whereas the pWRI-A has a wild type sequence. These RNAi vectors were introduced into non-*waxy* rice, *Oryza sativa* L. Japonica cv. Kinmaze ($Wx^b$), resulting 60 *WRI-A*/$Wx^b$ and 53 *WRI-B*/$Wx^b$ independent transgenic lines were used for following analyses.

**Table 1.** Silencing efficiency of R1 endosperm of *WRI*/$Wx^b$

| class | Estimated copy numbers of *WRI* genes | No. of silenced lines / total no. of *WRI*/$Wx^b$ lines | |
|---|---|---|---|
| | | *WRI-A* / $Wx^b$ | *WRI-B* / $Wx^b$ |
| I | 1 | 17 / 18 | 18 / 18 |
| II | 2～5 | 33 / 34 | 22 / 22 |
| III | 6～ | 8 / 8 | 13 / 13 |
| Total | | 58 / 60 | 53 / 53 |

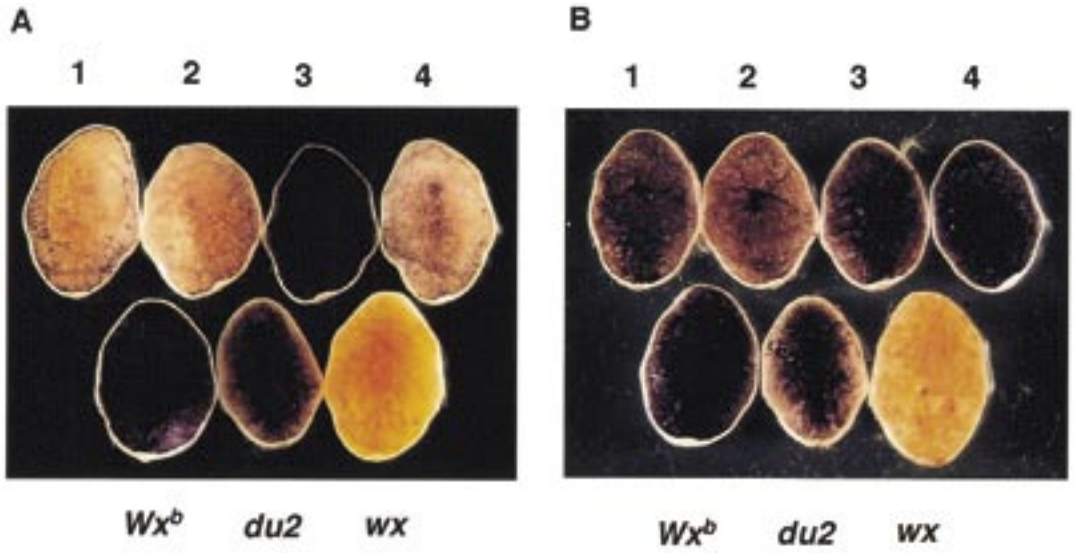


**Fig. 2.** Representative suppression phenotypes at the R1 seeds from *WRI*/*Wx*$^b$ transgenic rice. *WRI*/*Wx*$^b$ R1 seeds were sliced and stained by potassium iodide. **A**, R1 seeds from line WRI-A18 that carries a single copy of the *WRI-A* gene per diploid at R0 plant. **B**, R1 seeds from line WRI-B4 that carries a single copy of the *WRI-B* gene per diploid. Upper row, 1-4; R1 seeds from *WRI*/*Wx*$^b$ lines. Lower row, *Wx*$^b$; non-*waxy* rice (cv. Kinmaze), *du-2*; low amylose mutant *dull 2* (EM2), *wx*; *waxy* rice (cv. Musashimochi; *wx*$^{nmd}$),

***Estimation of transgene copy numbers***

To evaluate the quantitatively regulated RNAi effect on *Wx* expression, copy numbers of transgene at the *WRI*/*Wx*$^b$ plants were estimated by Southern blot analysis. Since both two vectors has a single recognition site of *Hin*dIII, genomic DNAs of *WRI*/*Wx*$^b$ plants were digested by *Hin*dIII and were used for Southern blot analysis. The result showed that the hybridization signal derived from endogenous *Wx*$^b$ appeared in 14.7 kbp (Fig. 1B *arrowhead*), whereas the *WRI* transgene signals appeared in other molecular size (Fig. 1B lane 1～4). *WRI* copy numbers were estimated from resulting the number of bands except the endogenous *Wx*$^b$. All *WRI*/*Wx*$^b$ lines were classified into 3 classes depending on the copy numbers of *WRI* gene (Table 1). Class I encompasses *WRI*/*Wx*$^b$ plants carried a single copy *WRI* gene, and 18 independent *WRI-A*/*Wx*$^b$ lines and 18 independent *WRI-B*/*Wx*$^b$ lines are categorized in Class I. Class II encompasses *WRI*/*Wx*$^b$ plants carried 2-5 copies of *WRI* genes and 34 independent *WRI-A*/*Wx*$^b$ lines and 22 independent *WRI-B*/*Wx*$^b$ lines are categorized into Class II. Class III encompasses *WRI*/*Wx*$^b$ plants carried over 6 copies *WRI* genes and 8 independent *WRI-A*/*Wx*$^b$ lines and 13 independent *WRI-B*/*Wx*$^b$ lines are categorized into Class III.

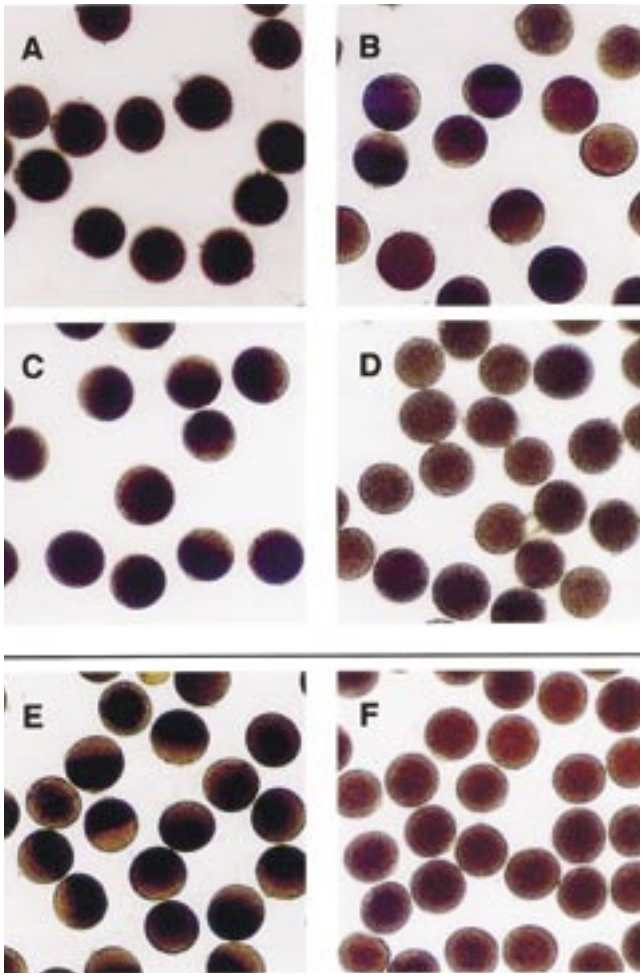


**Fig. 3.** Pollen phenotypes of *WRI*/*Wx*$^b$ transgenic plants.
Mature pollen grains were collected from anthers and stained by potassium iodide. Both the line WRI-A18 and WRI-B4 carry a single copy of transgene per diploid, whereas both the WRI-A42 and WRI-B39 carry the multiple copies of transgene per diploid. **A**, WRI-A18; **B**, WRI-A42; **C**, WRI-B4; **D**, WRI-B39; **E**, non-*waxy* rice (*Wx*$^b$: cv. Kinmaze); **F**, *waxy* rice (*wx*$^{nmd}$: cv. Musashimochi).

***High-frequency RNAi of $Wx^b$ was intiated by introduction of WRI genes.***

*Wx* promoter regulates pollen and endosperm specific *Wx* expression in rice, therefore the silenced *Wx* expression was analyzed at both pollen and endosperm in *WRI*/$Wx^b$ plants by iodine staining. When the *Wx* expression was completely silenced by RNAi, both pollen and endosperm show brown color as like as *waxy* (Fig. 2 *wx*, Fig. 3F). On the other hand, when the *Wx* expression was not completely silenced, pollen and endosperm show purple color as like as non-*waxy* (Fig. 2 $Wx^b$, Fig. 3E) or mid-colored phenotype as like as low amylose mutant *du*-2 (Fig. 2 *du-2*).

Ten R1 seeds from respective *WRI*/$Wx^b$ line were stained by potassium iodide and then the frequency of the RNAi on *Wx* expression was evaluated as follows. When the *waxy* or mid-colored endosperm was observed at least one seed among ten seeds of interest *WRI*/$Wx^b$ line, we defined that the event of RNAi was occurred at the *WRI*/$Wx^b$ line.

The silenced *Wx* phenotype was observed in 58 out of 60 *WRI-A*/$Wx^b$ lines (Fig. 2A, Table 1) and the 53 out of 53 *WRI-B*/$Wx^b$ lines (Fig. 2B, Table 1). These results showed that the both pWRI-A and pWRI-B led to efficient silencing of $Wx^b$ expression at more than 96% frequency. Furthermore, the high frequency of RNAi was observed in every class's *WRI*/$Wx^b$ line (Table 1 class I-III).

Among the transgenic lines carried a single copy of *WRI*, the pollen phenotypes were a purple ～mid-colored pollen at both *WRI-A*/$Wx^b$ (Fig. 3A) and *WRI-B*/$Wx^b$ (Fig. 3C) plants. Even though the class II and the class III *WRI*/$Wx^b$ lines carried multi-copy numbers of *WRI*, strong silenced phenotype was observed only at 8 out of 60 *WRI-A*/$Wx^b$ (Fig. 3B) and 3 out of 53 *WRI-B*/$Wx^b$ (Fig. 3D) plants. These results indicated that the pWRI-A and pWRI-B could lead to efficient RNAi in endosperms but not in pollen grains.

**Table 2** The proportion of *WRI*/$Wx^b$ lines which showed high heritability*1 of silenced phenotype

| Class | degree of silencing in R2 lines (%) | |
|---|---|---|
| | *WRI-A* / $Wx^b$ | *WRI-B* / $Wx^b$ |
| I | 66.7 | 27.8 |
| II | 61.8 | 68.2 |
| III | 100 | 76.9 |

*1 high heritability line is defined as the *WRI*/$Wx^b$ line which showed more than 75 % of silenced R1 endosperm.

***Effect of a mutation at 5′ splice site and gene dosage on RNAi of Wx.***

To examine the effect of a mutation at 5′ splice site on pWRI-B, the endosperm phenotype of the *WRI-B*/$Wx^b$ was compared with that of *WRI-A*/$Wx^b$. All Class I *WRI-B*/$Wx^b$ lines showed the mid-colored endosperm (Fig. 2B 1-3) like as *du2* endosperm (Fig. 2B *du2*), whereas 17 out of 18 Class I *WRI-A*/$Wx^b$ lines showed completely silenced *waxy* phenotype (Fig. 2A 1, 2 and 4). The results indicated that the suppression effect of *WRI-B* on $Wx^b$ expression was quantitatively lower than that of *WRI-A*. In both *WRI-A*/$Wx^b$ and *WRI-B*/$Wx^b$ lines, non-*waxy* endosperms were segregated (Fig. 2A 3, B 4). So the silenced state of $Wx^b$ was not heritable without *WRI* gene.

Cereal starchy endosperm is developed after double fertilization and its nuclear phase is triploid, therefore the gene dosage effect should be considered. When more than 75% of Class I endosperms showed *waxy* ～mid-colored phenotypes, a pollen-derived single *WRI* gene can be considered to suppress endogenous triplicate $Wx^b$ gene expression. Ten R1 seeds were tested and 66.7% of Class I *WRI-A*/$Wx^b$ lines showed over 75% of silenced endosperms, whereas only 27.8% of the *WRI-B*/$Wx^b$ lines were showed over 75% of silenced endosperms (Table 2). The result also supported that the suppression effect of the *WRI-B* was lower than that of *WRI-A*.

To confirm the quantitatively regulated RNAi effect, the levels of $Wx^b$ mRNA of *WRI-A*/$Wx^b$ were analyzed and compared with that of *WRI-B*/$Wx^b$ by northern blotting (Fig. 1C). As previous studies reported, only mature mRNA was detected in $Wx^a$ endosperm (Fig. 1C lane 1), whereas both 3.4 kb unspliced pre-mRNA and 2.3 kb mature mRNA were detected in non-transgenic $Wx^b$ endosperm (Fig. 1C lane 2). Compare to $Wx^b$ rice, both unspliced pre-mRNA and mature mRNA were reduced in either *WRI*/$Wx^b$ plants and the mature mRNA was completely degraded and could not be detected in *WRI-A*/$Wx^b$ endosperm (Fig. 1C lane 5). Compare to *WRI-A*/$Wx^b$ endosperm, mRNA was degraded but slightly remained in *WRI-B*/$Wx^b$ endosperm (Fig. 1C lane 6). The results indicated that the reduction of splicing efficiency by a G to T substitution mutation at 5′ splice consensus sequence can reduce the RNAi effect on *Wx* expression. The event of RNAi was quantitatively regulated and generated mid-colored endosperm phenotype.

***Small interfering RNAs in developing endosperm***

To confirm the RNA silencing was occurred by *WRI* gene, small RNA of developing endosperm was analyzed. Two kinds of small RNA molecules were detected in *WRI-A*/$Wx^b$ endosperm (Fig. 4A lane 2), whereas only one RNA species was detected in non-transgenic $Wx^b$ endosperm (Fig. 4A lane 1). So the lower RNA molecule (21～23nt) might be derived from both 5′ UTR of the *Wx* mRNA and a dsRNA from *WRI* gene. Hence the hybridization probe carries intron sequences (Fig. 1A Probe 2), there are possibility that the

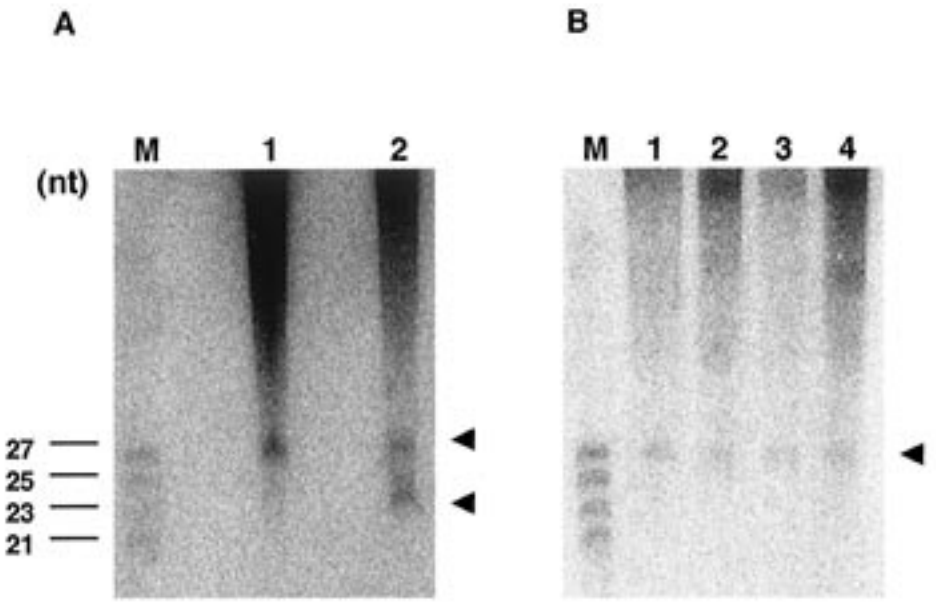


**Fig. 4.** Detection of siRNAs in developing endosperm of the *WRI/Wx*$^{b}$.
Total RNA was isolated developing endosperm and the 200 μg of total RNA was loaded on each lane. The probe 2 (see Fig. 1A) was used for siRNA detection. **A**, Detection of siRNA from R2 endosperm of a *WRI-A/Wx*$^{b}$ multiple copies line. M; Molecular size marker was loaded with the oligonucleotides 21, 23, 25, and 27mers, lane 1; non-transgenic *Wx*$^{b}$ rice (cv. Kinmaze), lane 2; *WRI-A/Wx*$^{b}$ line carrying with multiple copies of *WRI-A* genes. *Arrowheads* indicate small RNA signals. **B**, Control experiment was performed in four non-transgenic plants. M, Molecular size markers; lane 1, *Wx*$^{a}$ (cv. Labelle); lane 2, *Wx*$^{b}$ (cv. Kinmaze); lane 3, *wx*$^{nmd}$ (cv. Musashimochi); lane 4, *wx*$^{del}$ (a deletion mutant, 75KURwx4).

upper size of small RNA (25～27nt) was derived from spliced *Wx* 1st intron or a mobile element that inserted into *Wx* 1st intron (Umeda et al., 1991). Various non-transgenic rice, *Wx*$^{a}$, *Wx*$^{b}$, *wx*$^{nmd}$, and *wx*$^{del}$ were tested and *wx*$^{del}$ allele does not have any *Wx* sequences by large deletion (～20 kbp) of its chromosomal site. All non-transgenic rice showed upper size of small RNA species, even at the *Wx* gene was deleted (Fig. 4B lane 4). So the upper size of small RNA molecule might be derived from mobile element, but not derived from spliced *Wx* 1st intron and 'non-specific' small RNA.

## Discussion

Almost cereal genomes are largely duplicated by their polyploidy, and the genes are often functionally redundant, therefore the disruption of redundant gene function by mutation is difficult in such the polyploidy cereals as like wheat. Recently, the universal RNAi vectors are developed and are becoming useful tools for functional genomics, especially in polyploidy plants. However, the strong effect of RNAi vectors on gene disruption often lead to embryonic lethality and prevent the analysis of the gene that is functioned in the specific tissue and in a certain developmental stage. This study showed that the *Wx* promoter led to the endosperm specific RNAi and the mutation of consensus sequence of the intron spacer quantitatively reduced the RNAi effect on gene disruption. We propose that the spatial, temporal and quantitatively controlled RNAi will be useful to escape from embryonic lethality by strong RNAi effect on gene disruption and generate the various approaches for the functional genomics.

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# イネ *Wx* 遺伝子の胚乳特異的サイレンシングを引き起こすイントロンループ型 *Wx* 5'UTR RNAi ベクター

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**要　約**

胚乳特異的な遺伝子機能の破壊及び RNAi の量的調節について解明するため、胚乳特異的プロモーターで誘導され、第一イントロンをループに持つ RNAi ベクター、pWRI-A、pWRI-B を構築した。pWRI-B は第一イントロンの5' 側スプライシング部位に塩基置換があり、RNAi 特異的二本鎖 RNA の蓄積量が減少する。両方の RNAi ベクターとも96～100% の形質転換体で内在 *Wx* mRNA の発現抑制が観察された。さらに、*WRI-B/Wx*$^{b}$ の抑制効果は *WRI-A/Wx*$^{b}$ よりも弱かった。これらの結果により、*Wx* プロモーターが胚乳特異的な RNAi を誘導させることが明らかとなった。さらに、スプライス効率の調節が RNAi の抑制効果を制御できる可能性が示唆された。

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