

Epigenetically Fluctuating RNAi Efficiency Through Somatic Cell Divisions in a  
Unicellular Green Alga *Chlamydomonas reinhardtii*

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## Abstract

I intended to induce RNA interference (RNAi) in a unicellular green alga *Chlamydomonas reinhardtii*, which is aimed at *aadA* mRNA (conferring spectinomycin resistance). To achieve this purpose, an artificial inverted repeat DNA (silencer DNA construct) was designed to generate a hairpin RNA of long stem and short loop, and single copy of it was introduced into a strain expressing the *aadA* mRNA stably.

I succeeded to induce the RNAi, however after succeeding cell divisions, the induced RNAi efficiency was varied prominently among clones without accompanying prominent genetic changes in the silencer construct. Northern blot analyses showed reverse correlation between the detected amount of the hairpin RNA and the spectinomycin resistance. Therefore, observed inefficient RNAi is most probably due to the limited amount of the available hairpin RNA that is to be processed into siRNA. This also suggests that *C. reinhardtii* has no system to make secondary siRNA *via* double stranded target mRNA generated by RNA dependent RNA polymerase.

Moreover, another reverse correlation was detected between the efficiency of silencing and the frequency of methylated CG (\*CG) in the silencer region, i.e., the \*CG frequency was about 15% for highly silenced sub-clones, while it was about 53% for weakly silenced sub-clones. Even for the promoterless inverted repeat (IR) construct,

\*CG was accumulated almost to the equivalent level that is in the strongly silenced sub-clones. This shows accumulated \*CG in the IR region is primarily due to the intrinsic structure of the region, and transcription of the region might enhance the \*CG frequency. Suggesting *C. reinhardtii* has no system for siRNA dependent DNA methylation, \*CG accumulation was not observed in the target *aadA* gene.

Treatment of RNAi induced cells by a histone deacetylase inhibitor (Trichostatin A ) increased the accumulated amount of the hairpin RNA rapidly. In addition to that, the reagent affected the elongation step of the transcription: the ratio of fully transcribed IR transcripts to the various 3'-truncated IR transcripts was increased about threefold by the reagent. Therefore, highly likely transcription of the silencer construct was repressed by \*CG related epigenetic silencing mechanisms at the elongation step as well as at the initiation step. Furthermore, \*CG might be not so faithfully maintained through DNA replications in this organism. These are the probable main reasons for the fluctuating RNAi efficiency even among sub-clones of a RNAi-induced cell after succeeding somatic cell divisions.

## **Introduction**

Epigenetic gene repressions are divided into two types depending on the period of their exertions. Many of them act at the transcriptional steps, while some work at the post-transcriptional steps. RNAi is one of the typical examples of post-transcriptional repressions, because primarily it achieves repression by degradation of a specific mRNA in a cytoplasm. On the other hand, heterochromatin formation is one of the typical examples of transcriptional repression.

Long double stranded RNA (dsRNA) acts as an efficient trigger molecule for RNAi induction. The core reactions of RNAi seem common among various eukaryotes, i. e., double stranded or hairpin RNA is recognized by cytoplasmic RNase III-like enzyme (Dicer), and this enzyme degenerates the long dsRNA into 21-26 nt dsRNA of 3'-2 nt overhang. One of the two strands (siRNA) is transferred into an effector complex named RISC (RNA induced silencing complex), which includes a small RNA binding protein named argonaute to digest a target mRNA molecule. A siRNA embracing RISC searches the target mRNA by nucleotide sequence dependent manner, and digests it at the middle the complementary sequence of the siRNA. This mechanism can be induced artificially by various procedures, e.g., infection of remodeled viral vector, injection of exogenously synthesized siRNA or long double stranded RNA into cytoplasm, and

transformation with DNA construct for providing hairpin shape RNA.

RNAi is an evolutionarily well conserved mechanism. This mechanism was discovered in *D. melanogaster* for the first time in eukaryote species. At the present time, it is well known that RNAi mechanism exists in almost all model organisms of eukaryotes. However, accessory responses concomitantly occur with the core RNAi reactions, are not identical among eukaryotes. In the case of *C. elegans* and *A. thaliana*, they have a pathway to convert the target mRNA into dsRNA by siRNA primed RNA dependent RNA polymerase (RdRP). The newly generated dsRNA is also recognized by Dicer to produce a large amount of secondary siRNA. Generation of secondary siRNA leads to the efficient degeneration of the target mRNA, which lasts for a long period stably. Moreover, in some eukaryotes, methylation occurs specifically at the DNA sequence that is complementary to the siRNA. Such siRNA dependent *de novo* methylation is called RNA-directed DNA methylation (RdDM). Generally, cytosine methylation is predominantly detected in symmetric CG sequence, because only methylation at the site is efficiently inherited to daughter cells by maintenance type methyltransferases. In land plants, cytosine methylation at CHG sites (where H=A, T, or C) is faithfully inherited to daughter cells in addition to the CG sites (Cerutti H, 2003; Tariq M and Paszkowski J, 2004; Chan *et al.*, 2005).

Strung nucleosome beads are condensed in a nuclear compartment, which are known as chromatin. Euchromatin and heterochromatin are typical example of chromosome domain that differs in condensed degrees and sizes of composed nucleosomes. In euchromatic region, nucleosomes are loosely condensed, therefore transcription facilitate factors are able to easily access to genes. On the other hand, in heterochromatic region, nucleosomes are densely packaged and comprising higher order structure. Generally, heterochromatic chromosome region is transcriptionally inert and contains very few expressed genes and a large number of repetitive DNA elements, such as transposons.

N-terminal regions of all histone proteins protrude from core domain of nucleosome and are competent to various modifications such as methylation, phosphorylation, acetylation, ubiquitination, and so on. Histones located at the heterochromatin domains are featured by peculiar modifications, such as methylation, at the specific amino acid residues of the histone tails. Particularly, histones composing heterochromatic region are severely deacetylated. Moreover, the DNA in heterochromatin is often supplemented with CG methylation. While histones located at euchromatic regions usually have different types of modifications, such as acetylation and DNA in the region is not methylated. Therefore, DNA methylation, histone

modifications, and heterochromatin formation seem closely related (Loidl P, 2004).

In *A. thaliana*, more than 30 % of total C's in the genome are methylated (Adams RL *et al.*, 1990), while methylated cytosine is limited to only 0.7% and 1.1 % in the case of a unicellular green alga *C. reinhardtii* (Hattman S *et al.*, 1978) and its closely related multicellular green alga *Volvox carteri* (Babinger P *et al.*, 2001), respectively. It remains unknown how such a limited amount of methylated cytosines (\*C) contribute to the transcriptional repression in these organisms. It has been reported that methylated cytosine is not accumulated in a retrotransposon *TOC1* and DNA a transposon *Gulliver*, while *REMI* retrotransposon of long terminal repeat is highly methylated in *C. reinhardtii* (Jeong BR *et al.*, 2002; Zhang C *et al.*, 2002; Perez-Alegre M *et al.*, 2005).

Taking advantage of efficient transformation for the nuclear (Kindle *et al.*, 1989), chloroplasts (Boynton *et al.*, 1988), and mitochondria (Randolph-Anderson *et al.*, 1993 Yamasaki T *et al.*, 2005), *C. reinhardtii* has been used as a model organism to study various aspects of cell biology such as photosynthesis, organelle biogenesis, and flagellar assembly. Moreover, the genome of *C. reinhardtii* has been already nearly completely sequenced. This result shows existence of numerous unidentified genes in the genome, which including interesting functional domain or homologous to known gene occasionally. These newly defined genes may indicate new insight into biological

process by experiments using reverse genetics approaches such as insertional mutagenesis, tilling, and targeted gene disruption. Above all these approaches, RNAi mediated gene knock down method is used as one of the most convenient approach. Recently, bacterial *aadA* gene that confers spectinomycin resistance is utilized successfully for nuclear transformation of *C. reinhardtii* as well as traditional autotrophic markers, such as *NitI* (Cerutti *et al*, 1997 (a); Kindle KL *et al*, 1989). To achieve high level expression of non-endogenous genes, several intact or modified endogenous promoters have been tested (Leon-Banares R *et al.*, 2004). Among them, the promoter for *RbcS2* has prominent advantage of being fully characterized for the essential regions (Victoria *et al.*, 1998). By virtue of efficient nuclear transformation and improved promoters, diverse *C. reinhardtii* endogenous genes have been silenced by transcribing an inverted repeat DNA constructs (silencer DNA construct) of which transcripts are designed to be holed into hairpin structure in the cytoplasm. However, it is often reported that silencing efficiency shifts to the lower along with the period of cell culture without rearrangement of the silencer DNA construct (Schroda M, 2006). It remains unknown why RNAi triggered by the expression of an artificial IR construct will not lost long.

In this study, I induced RNAi by expressing the IR DNA construct, which is



aimed at the *aadA* mRNA. I closely analyzed the time source change of the RNAi efficiency accompanied by succeeding somatic cell divisions.

## Materials and Methods

### *Strains and culture conditions*

*C. reinhardtii* 19-P(1030) is generated from the wild type strain cc-124 (mt-) by introducing single P-679 plasmid, which is carrying an exogenous *aadA* gene driven by *C. reinhardtii* *RbcS2* promoter, into the nuclear genome (Cerutti H *et al.*, 1997 (a)). This strain was obtained from *Chlamydomonas* Genetic Center (c/o Dr. Elizabeth H. Harris, Department of Botany Duke University Durham, North Carolina 27706, USA). Single colony isolation was carried out using agar plate of Tris-Acetate-Phosphate (TAP) containing 90µg/ml spectinomycin. Several colonies of faster growth were picked up and mixed together to start liquid culture for further transformation to induce RNAi aimed at *aadA* mRNA.

### *Composition of a silencer DNA construct*

To induce RNAi aimed at the *aadA* mRNA, most part of the *aadA* gene ORF sequence (omitting five codons for the N-terminal) was arranged to make a tail-to-tail inverted repeat with a short hinge to connect them. For the hinge, 79 bp of *cox2-no2-*

intronic DNA sequence was used (Watanabe KI and Ohama T, 2001). To drive the inverted repeat construct, endogenous *RbcS2* 5'-UTR sequence was connected at its upstream region as a promoter, while endogenous *RbcS2* 3'-UTR sequence was ligated at the downstream region as a terminator.

Therefore, this silencer construct was composed of five segments, i. e., *RbcS2* 5'-UTR, sense-strand of *aadA* gene, *cox2-no2*-intronic DNA, antisense-strand of *aadA* gene, and *RbcS2* 3'-UTR. Four of the five components were prepared by the PCR method using appropriate templates and a set of primers. Only *cox2-no2*-intronic DNA was chemically synthesized. Used primers were summarized in Table 1. Segments for *RbcS2* 5'-UTR, sense-strand of *aadA*, and *cox2-no2*-intronic DNA were integrated into one by the Megaprimer PCR method (Molecular Cloning, CSHL PRESS), and then it was cloned into the *Eco* RI/*Not* I site of pSP124S bearing *ble* gene to confer zeocin resistance (Lumbreras V *et al.*, 1998). I named this as front-silencer half/pSP124S. Segments for antisense-*aadA* and *RbcS2* 3'-UTR were also fused by the Megaprimer method, and then cloned into *Not* I/*Sac* I site of the front-silencer half/pSP124S. Accomplished silencer DNA construct was schematically shown in Fig. 1.

#### *Culture conditions and transformation procedures*

Unless noted otherwise, cells used for transformation were grown mixotrophically in liquid TAP medium under moderate and constant white fluorescent light ( $20 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) at  $25^\circ\text{C}$  with vigorous shaking. Silencer plasmid DNA was linearized by *Kpn* I, and used to transform 19-P(1030) using biolistic particle delivery system (PDS-1000/He, Bio Rad). Transformants were directly selected on TAP-agar plates containing  $10 \mu\text{g/ml}$  zeocin. Plates were incubated under white fluorescent light  $20 \mu\text{mol m}^{-2} \text{s}^{-1}$  (a 16:8 hour light : dark regimen) at  $25^\circ\text{C}$  for colony formation.

*Spotting test to estimate the level of spectinomycin resistance*

Independent transformants were cultured to reach stationary phase in TAP medium, and then subjected to tenfold dilution with TAP medium. Five  $\mu\text{l}$  of the diluent were spotted on the various concentrations of spectinomycin plates, and incubated for 7-10 days under white fluorescent light  $20 \mu\text{mol m}^{-2} \text{s}^{-1}$  (a 16:8 hour light:dark regimen) at  $25^\circ\text{C}$ . Relative growth rates were judged by eyes based on the greenish color of the spots.

### *Spectinomycin resistance assay for RNAi induced transformants*

TAP culture of RNAi induced transformants were spread on TAP-agar plates at low density, then appeared colonies were cultured independently in 3 ml of TAP medium until to reach stationary phase. For each culture, the level of spectinomycin resistance was assayed by the spotting test described above.

### *Preparation of nucleic acids*

To prepare genomic DNA, cells grown to logarithmic phase were pelleted by centrifugation, and suspended in 1/5 volume of TEN wash buffer [10 mM Tris-HCl (pH 8.0), 10 mM EDTA (pH 8.0), 150 mM NaCl]. Then, washed cells were repelleted by centrifugation. Total DNA was isolated from the pellet by the CTAB method (sambrook and Russell, 2001). Total RNA was prepared using TRIZOL reagent (Invitrogen) following the manufacture's instruction. Mixture of RNaseA and RNaseT1 (RNaseA/T1) was used to digest single strand RNA. I used RPAIII Ribonuclease protection kit (Ambion) for this treatment. To concentrate small RNAs, High molecular weight RNAs were precipitated with 10 % PEG-0.5M NaCl mixture (final

concentration), and remained small RNAs were purified from the supernatant using QIAGEN RNA/DNA midi kit (QIAGEN).

## **DNA/RNA blotting and hybridization procedures**

### *Preparation of DNA and RNA blotted filters*

To prepare the membranes for Southern hybridization, total DNA was completely digested with appropriate restriction enzymes, resolved on 0.8 % agarose gel, and then blotted onto a positive charged nylon membrane (Hybond N+, GE Healthcare Life Science) by capillary transfer using alkali buffer (0.4M NaOH, 1M NaCl). To prepare the membranes for Northern hybridization, RNA was separated by formaldehyde containing agarose gel, and then blotted onto the nylon membrane using a semi-dry electrobloter. To fix the RNA molecules on the filter, UV cross-link and baking (for 2 hours at 80°C) were carried out. To separate small RNAs, 15 % polyacrylamide/8M urea gel was used.

### *Preparation of digoxigenin (DIG) labeled probes*

DIG labeled DNA probes: All DIG labeled DNA probes were prepared using PCR DIG Probe synthesis kit (Roche). I prepared four kinds of DIG labeled DNA probes (Table 2) for Southern and Northern hybridization as described below.

DIG labeled RNA probes: I prepared five kinds of RNA probes for Northern hybridization. DIG-conjugated UTP was incorporated transcriptionally using DIG RNA labeling mix (Roche). For the reaction, DNA fragments carrying T3 or T7 RNA polymerase promoter were prepared by the PCR method, or appropriate fragments prepared by the PCR method were cloned into pBluescript II SK (-) vector that has T3 and T7 RNA polymerase promoters. PCR primers to prepare these DNA fragments were listed in Table 3.

#### *Hybridization and wash conditions*

Hybridization experiments were carried out using high SDS hybridization buffer (Roche), anti-DIG-AP fab fragment (Roche), and CDP-star (Roche), following the manufacture's protocol. DNA probe hybridization and high stringency wash were

carried out at 40 °C. Hybridization with RNA probe and following high stringency wash were carried out at 68 °C. To detect siRNA, hybridization in high SDS hybridization buffer and following wash with high stringency were carried out with 2× SSC [1× SSC contains 0.15M NaCl and 0.015M sodium citrate] at 35 °C. Signals were detected by a luminescent image analyzer, LAS-1000 (Fuji Film).

#### *DNA digestion with methylcytosine sensitive restriction enzymes*

To examine the methylation status at the CCGG sites, I used isosyzomeric restriction enzymes, *Hpa* II and *Msp* I. These two enzymes recognize the same target sequence CCGG but differ in their sensitivity to methylcytosine. *Hpa* II dose not cleave \*CCGG (\*C=5'-methylcytosine) and C\*CGG, whereas *Msp* I is able to cleave C\*CGG but not \*CCGG. Flanking sequences of the recognition site can affect the cleavage. GGC\*CGG is cleaved at exceptionally slow rate by *Msp* I. Digested samples were subjected to electrophoresis to separate the resulted fragments.

#### *Bisulfate genomic PCR*



Genomic DNA was completely digested with 10 kinds of restriction enzymes, i. e., *Sac* I, *Kpn* I, *BbrP* I, *BamH* I, *Bgl* II, *Xba* I, *Eco22T* I, *Pst* I, *Mlu* I, and *Xho* I to analyze the position of methylated C's in the *aadA* IR region. While for analysis of methylated C's in the *aadA* gene, genomic DNA was completely digested with *Sac* I and *Kpn* I. Digested DNA was treated with chemical reagents to convert C to U leaving \*C as it is (Trygve, 2004). Nested PCR was carried out to amplify the region to be sequenced. PCR primers used for the nested PCR were listed in Table. 4. I used Y (Y = C or T) instead of C for the forward primers when C was in the context of CpG in the original sequence, and R (R = A or G) instead of G for the reverse primers when G was in the context of CpG in the original sequence. PCR fragments were cloned into pT7 Blue Vector (Novagen) by TA-cloning, and at least 8 clones were sequenced for each kind of the samples.

#### TSA treatment

Trichostatin A (TSA) treatment was carried out for cultures in the middle logarithmic phase. TSA/ethanol stock solution (5 mg/ml) was added to the cell culture (final concentration 50 ng/ml).

## Results

### *Inducement of RNAi aimed at exogenous aadA mRNA*

To induce RNAi aimed at *aadA* mRNA, I transformed 19-P(1030) that is stably expressing the *aadA* mRNA, with a linealized silencer/pSP124S plasmid carrying *ble* gene as a transformation marker (Fig. 1). Transformants were selected based on zeocin resistance. When RNAi-mediated suppression was activated successfully, slow growth of transformants on the spectinomycin plates were expected as a result of decreased aminoglycoside-3''-adenyltransferase that is the product of *aadA* gene.

First screening was carried out for randomly chosen 59 zeocin resistant transformants, then their growth rates were assayed using agar plates of various spectinomycin concentrations. Forty of 59 transformants could not grow or evidently showed slower growth than parental 19-P(1030) strain on TAP agar plates containing 250 µg/ml spectinomycin (spc250/TAP), while all of them survived on the spc100/TAP-plates on which wild type *C. reinhardtii* can not grow. However, 36 of 40 transformants showed prominently slow growth even on non-selective TAP agar plates as well as the spectinomycin containing plates. Remained four transformants showed

prominent slow growth specifically on spectinomycin plates. These four transformants were estimated as promising candidates for RNAi induced transformants, and I named them as RNAi-13, -17, -18, and -37, respectively. These four strains could not survive on *spc400*/TAP-plates on which parental strain 19-P(1030) grows as fast as on TAP plates (Fig. 2). For these four transformants, I analyzed the integrated copy number of the silencer plasmid by Southern hybridization and also the integrated region of the plasmid by PCR analyses. Southern hybridization showed RNAi-13 was carrying single silencer plasmid, while PCR analyses cleared that some part of inverted repeat was not integrated in the genome. RNAi-17 and -18 carried three and four copies of the plasmid, respectively. In contrast, RNAi-37 was carrying the single intact silencer construct and also the marker gene *ble* (data not shown). Therefore, this transformant was used mainly for further experiments. I confirmed the existence of siRNA and *aadA* hairpin RNA to prove that decreased spectinomycin resistance of RNAi-37 was due to inducement of RNAi aimed at *aadA* mRNA.

I detected the signal of approximately 1.6 knt long RNA molecule (Fig. 2B), which size was consistent with the expected intact transcript of the silencer construct that was composed of 770 nt long complete inverted repeat hinged by a 79 nt long intron, and also the signal for about 24 nt long siRNA molecule from the small RNA

fraction (Fig. 2C). Therefore, I concluded that reduction of spectinomycin resistance observed for RNAi-37 was due to inducement of RNAi aimed at *aadA* mRNA.

To determine the genomic locus of the integrated plasmid, I amplified the flanking genomic region of the plasmid by the RESDA-PCR method (Gonzalez-Ballester D *et al.*, 2005). BLAST search using the obtained sequence showed that the plasmid was inserted at the scaffold\_3: 1422072 (<http://genome.jgi-psf.org/> ver. 3.0; Grossman AR *et al.*, 2003). The neighboring regions were rich in genes. Therefore, the silencer DNA construct was most probably integrated in an euchromatic region of the genome.

#### *Fluctuation of spectinomycin resistance by succeeding somatic cell divisions*

I noticed that colonies appeared on spc100/TAP-plates were varied in their sizes, when a small portion of RNAi-37 liquid culture was spread (Fig. 4A). Showing remarkable contrast, 19-P(1030), which is the parental strain of RNAi-37, formed even size of colonies on the same kind of plates (Fig. 4A).

To analyze this phenomenon in detail, I randomly picked up 6 of RNAi-37 colonies, which were raised by single colony isolation of a RNAi-37 colony using

non-selective TAP-plates. I numbered the clones numerically 1 through 6, and analyzed their relative spectinomycin resistance by the spotting test (Fig. 4). Phase I spotting test was carried out as follows: each one of 6 clones was grown to stationary phase in non-selective liquid TAP-medium independently, then tenfold diluent (5 $\mu$ l) was spotted on spc50/TAP, spc100/TAP, spc200/TAP, spc300/TAP, ampicillin (amp)100/TAP, and TAP without acetate-plates, then incubated for 10 days. Various growth rates were observed for the spots on spc50/TAP, spc100/TAP, spc200/TAP-plates, while their growth showed no prominent difference on the TAP and TAP without acetate plates (Fig. 4C). They were ranged by the sensitivity for spectinomycin, as clone-2, -1, -3, -4, -5, and -6, from the highest to the lowest. Then, each clone was subjected to totally about 20 times of somatic cell divisions in TAP medium by succeeding transplantation of it at the middle logarithmic growth phase. Then, second colony isolation was carried out by spreading small portion of the culture on TAP-plates. After 10 days, 10 colonies for each clones were randomly picked up, and cultured in 2 ml of TAP medium until to reach stationary-phase. Then, totally 60 sub-clones (10 sub-clones from each one of 6 kinds of RNAi-37 clones) were subjected to the spotting test using various kinds of plates.

Non of 60 sub-clones showed substantial difference for their growth on

amp100/TAP, zeo50/TAP, zeo200/TAP, and TAP-without acetate-plates, while notable difference of growth were observed again even among the 10 sub-clones originated from the single clone. Actually, 10 sub-clones derived from the clone-2, i. e., sub-clone 2-1 through 2-10, showed various growth rates on spc100/TAP-plates, while all of them could not grow on spc200/TAP plates (Fig. 4C). On the other hand, 10 sub-clones derived from the clone-5, i. e., sub-clone 5-1 through 5-10, showed similar growth rates on spc100/TAP-plates, while they showed various growth rates on spc200/TAP-plates (Fig. 4C). Close analysis of 60 sub-clones showed that some of them were shifted to slightly higher spectinomycin resistant cells, while many of them did to a bit lower resistant cells with variegation even among sub-clones raised from single clone. On the other hand, sub-clones of 19-P(1030), which were subjected to the same treatment executed to RNAi-37, showed no detectable difference for the growth rates on all kinds of spectinomycin plates ( growth on a spc400/TAP-plate was shown in Fig. 4D).

For the above 60 sub-clones, I confirmed that no extensive rearrangements occurred at the inside or bordering regions of the *aadA* gene and the silencer construct by the Southern hybridization analyses. This suggested that fluctuating spectinomycin resistance was due to epigenetic responses. A series of treatments described above were also carried out for RNAi-13, -17, and -18. Similar phenomenon, i. e., prominent

fluctuation of spectinomycin resistance among sub-clones was observed (data not shown).

In addition to the epigenetic sensitivity change for spectinomycin, prominent recovery of spectinomycin resistance owing to genetic changes was also detected. Cells bearing such changes were easily distinguished from the epigenetic ones, because they recovered the spectinomycin resistance to the level of the parental cell 19-P(1030), i.e., 400 µg/ml spectinomycin . I analyzed four clones that grow even on spc400/TAP-plates, which was obtained after succeeding liquid culture. Southern hybridization analysis with *Eco*TI4 I digestion showed that these four contained new *Eco*TI4 I DNA fragments of the silencer region (Fig. 5). Further analyses by the PCR method showed, two of the four clones were sharing the same deficiency in the IR region, while the other two had mutually different kinds of deficiencies at the IR region. Therefore, totally three kinds of deficiencies were detected from four fully spectinomycin resistance recovered clones.

*Correlation between the level of spectinomycin resistance and the amount of accumulated hairpin RNA and siRNA*

I analyzed the relative amount of hairpin RNA and siRNA for three RNAi-37 sub-clones of high spectinomycin resistance (#4-#6 in Fig. 6A) and also three of low resistance (#1-#3 in Fig. 6A) by Northern blot analyses. Using the DIG-labeled sense-*aadA* RNA as a probe, a clear band was detected at the position of about 1.6 knt long from all of the sub-clones, which was the expected size of intact silencer DNA transcript. It was prominent that the detected 1.6 knt signal from the high resistant sub-clones (#4-#6) were constantly weaker than from the low resistant ones (#1-#3) (Fig. 6B). By treatment of RNase A/T1 mixture, the 1.6 knt band sifted to the position corresponding to the half of the original molecular weight. This showed the 1.6 knt RNA was held into hairpin form of long double stranded region in the cytosol as intended.

Detection of siRNA in the small RNA fraction was carried out using the small RNA fractions separated on 15 % polyacrylamide gel. Much stronger signal of the siRNA molecules was detected from the weaker resistant sub-clones (#1-#3) than from the high resistant sub-clones (#4-#6) by sense-*aadA* RNA probe (Fig. 6B). Positive correlation between the amount of siRNA and the hairpin RNA was also clear. Therefore, it seemed that the primary limiting factor for RNAi efficiency was the amount of silencer hairpin RNA.



### *Analysis of 5-methylcytosine in the inverted repeat DNA region*

I estimated the frequency of 5-methylcytosine (\*C) by Southern blot analyses and the genomic bisulfate sequencing method. To assess the \*C frequency in the CCGG sequence located in the silencer DNA construct and the *aadA* gene that codes the target mRNA, I prepared total DNA from two strongly silenced and two weakly silenced sub-clones of RNAi-37.

### *Detection of 5-methylcytosine by Southern blot analysis using methylation sensitive isoschizomers*

First, I completely cleaved these genomic DNA with *Eco*T14I that is insensitive to \*C to arrange the expected detectable DNA fragments at appropriate sizes. Subsequently, the DNA sample was divided in two, and one was further digested with *Hpa* II and the other with *Msp* I. Existence of \*CpG in CCGG sites of the *RbcS2* promoter region, which to drive silencer DNA construct, *ble* and *aadA* genes, was assayed by P-probe (Fig. 10A). On the other hand, C-probe was utilized to detect \*CpG

in CCGG sites located in the *aadA* gene and *aadA* IR region (Fig. 10A).

Seven detectable DNA fragments by P-probe, i.e., 482 bp, 116 bp, 84 bp, 82 bp, 74 bp, 73 bp, and 62 bp long, are expected from the DNA sequence of the construct when all CCGG sites in the *RbcS2* promoters and *aadA* IR are completely digested. If \*CCGG or C\*CGG sites were accumulated abundantly in the *RbcS2* promoter regions, *Hpa* II digestion should give signals of which sizes are corresponding to the combined length of the neighboring fragments. However, I could not detect such signals. Only the band of 482 bp long, which corresponded to the completely digested *RbcS2* promoter of the *aadA* IR, was observed irrespective of the strength of RNAi (Fig. 10B). This indicated that existence of \*CCGG and C\*CGG sites were under detection level, if any. On the other hand, irrespective of the RNAi efficiency, the 482 bp signal detected from *Msp* I digested samples was constantly stronger than that from *Hpa* II digested samples. This indicated C\*CGG was slightly accumulated in the promoter region.

Using C-probe, I carried out the analysis of \*C accumulation at the restriction sites in the *aadA* gene and *aadA* IR region (Fig. 10A). According to the DNA sequence, 520 bp and 372 bp fragments are the detectable bands when these regions are digested completely by *Hpa*II or *Msp*I. The former band is the originated from the *aadA* gene, while the latter is the originated from the *aadA* IR. Showing the existence of very few, if

any, C\*CGG and \*CCGG sites in the *aadA* gene only 520 bp band was detected from all samples. In addition to that strength of the signal had no detectable difference due to the kinds of restriction enzymes used and origin of the genomic DNA, i. e., whether DNA was prepared from strongly silenced clones or weakly silenced ones. This showed there were no detectable accumulation of \*C in the CCGG sites of the *aadA* gene.

For strongly silenced two clones, the density of the 372 bp band was same irrespective of the restriction enzymes used. On the other hand, for weakly silenced two clones, the signal was prominently stronger for *Msp* I digested samples than *Hpa* II digested ones (Fig. 10C). This showed significant and specific accumulation of C\*CGG in the weakly silenced clones. Moreover, showing the significantly accumulated C\*CGG in the *aadA* IR region of the weakly silenced sub-clones, a signal that corresponds to unresolved 553 bp and 605 bp bands were specifically detected for *Hpa* II digested samples of which genomic DNA was prepared from weakly silenced sub-clones. These signals were result of incomplete digestion at some CCGG sites.

The 424 bp band was also specific for weakly silenced clones. Considering the DNA sequence of the *aadA* IR region and result of the bisulfate genomic sequencing (see below), the 424 bp band was highly likely due to the slow digestion of *Msp* I for the GGC\*CGG sequence, but it did not owe to the existence of \*CCGG. Altogether,

Southern blot analyses showed that very slightly accumulated C\*CGG in the *RbcS2* promoter region and significant accumulation of C\*CGG at the *aadA* IR silencer region.

#### *Detection of methylated cytosine by the bisulfate genomic sequencing method*

I analyzed the frequency and location of the \*C in upper strand of the first half of the *aadA* IR region by the bisulfate genomic sequencing method (Liu, L *et al.*, 2004).

Methylated cytosines were detected within the limits of CpG context. On average, \*CpG frequency was 15.3 % for the analyzed 59 CpG sites in the front half of the *aadA* IR region of the strongly silenced clones, while it reached 53.4 % for weakly silenced clones (Fig. 8). The same method was applied to the equivalent region of the *aadA* gene that codes the target mRNA. For this gene, \*CpG was virtually absent (below 1 % , data not shown). This showed *C. reinhardtii* probably does not have siRNA mediated trans-acting cytosine methylation pathway aimed at the homologous DNA region. It is notable that distribution of \*CpG was prominently biased to be rich in the region close to the *RbcS2* promoter, and had the tendency to decrease gradually along with the direction of its transcription.

### *Relationship between the frequency of \*CpG and aadA IR transcription*

To determine whether accumulation of \*CpG in the *aadA* IR region depends on transcription, I made an *aadA* IR DNA construct bearing no promoter and terminator, but retaining the other region as it was in the original silencer plasmid. This construct was introduced into 19-P(1030). I selected two transformants, which were carrying single intact promoter less *aadA* IR construct and *aadA* IR transcript was under detectable level by Northern hybridization (Fig. 9). For these two transformants, the frequency of \*C in the CpG sites located in the corresponding region analyzed for the original *aadA* IR was 20.1 % for one transformant, while it was 18.7 % for the other transformant. These frequencies were roughly equal to the average of \*CpG frequency for strongly silenced clones of RNAi-37 (i. e., 15.3 %). This indicated that transcription was not absolutely necessary for \*CpG accumulation. Therefore, highly likely intrinsic IR structure is the primary determinant of the CG-methylation in the *aadA* IR region.

### *Effect of TSA treatment for the accumulation of hairpin RNA and siRNA*

There was a clear reverse correlation between the amount of accumulated hairpin

RNA and the frequency of \*CpG, i. e., less amount of hairpin RNA was detected from the sub-clones that carry heavily methylated *aadA* IR construct. To study whether transcription activity for *aadA* IR is affected by histone modifications, I analyzed the effect of Trichostatin A (TSA) that is a histone deacetylase inhibitor. One strongly silenced and one weakly silenced RNAi-37 sub-clone cells were treated with TSA (final concentration 50 ng/ml) at the logarithmic growth phase in liquid culture. Same amount of culture liquid was withdrawn periodically during the treatment. Total RNA was prepared from these samples and quantity of the hairpin RNA and siRNA were compared by Northern blot analyses.

After 20 to 40 minutes of incubation with TSA, increase of the hairpin RNA was observed prominently for both types of the sub-clones (Fig. 10A). In the case of strongly silenced sub-clones, increase of the hairpin RNA reached to the max at 40 minutes incubation, while in the case of weakly silenced sub-clones, increase of the hairpin RNA was very slow and it reached the max at 120 - 180 minutes (Fig. 11).

Contrasting to the prominent effect on the accumulated amount of *aadA* IR transcript, no obvious effect was detected for the transcript of the endogenous *RbcS2* gene, which was analyzed as a control (Fig. 10A). Therefore, the increased amount of the *aadA* IR transcript was highly likely the results of specifically enhanced activity of

the transcription that had been repressed by deacetylated histone related mechanisms.

I also examined the effect of TSA for the production of siRNA. Total RNA was prepared from the cells cultured in the TSA containing TAP medium at the treatment period of 90 and 180 minutes, and also from the cells that were cultured for 180 minutes in the TSA/TAP medium and then washed and re-cultured for additional 180 minutes in the TAP without TSA (180 TSA/180 TAP cells). Increase of siRNA was observed for both types of sub-clones. The amount of siRNA detected from 180 TSA/180 TAP cells were substantially increased than that detected from 180 min culture in TSA. Increase of siRNA by TSA treatment was featured as follows: (i) Increase had one to two hours delay to that of the hairpin RNA, and (ii) the increase was relatively slow compared with the hairpin RNA.

#### *Effect of TSA treatment for elongation step of the aadA IR transcription*

Not mutually exclusive two types of mechanism are plausible for the *aadA* IR repression: (i) initiation of the transcription is repressed, or (ii) elongation step of the transcription is repressed. To assess the extent of the latter effect, I carried out Northern hybridization to detect prematurely terminated transcripts and also complete one using

*aadA* sense- and anti-sense RNA probes. Northern hybridization analyses were carried out for five RNAi-37 sub-clones of which RNAi efficiencies were diverse widely (Fig. 11A).

When hybridization was carried out using sense-*aadA* RNA probe (750 base long), which hybridizes to the most part of the latter-half hairpin transcript, trifling smear signals corresponding to 1.0 to 1.6 knt long incomplete transcripts were detected besides 1.6 knt long complete hairpin RNA (Fig. 11B). Using the same membrane, another hybridization was carried out with antisense-*aadA* RNA probe (620 base long), which hybridizes to most part of the front-half of the hairpin transcript. Strong smear signals corresponding to 0.5 to 1.6 knt long incomplete *aadA* IR transcripts were detected (Fig. 11B). Signal intensity for the complete 1.6 knt transcript was apparently same irrespective of which one of the two RNA probes was used, while smear signals were much strongly detected by the antisense-*aadA* probe than sense-*aadA* probe (Fig. 11B). The ratio of incomplete transcripts (ICP) against the complete transcripts (CP), i. e., (ICP/CP) had clear correlation with the RNAi efficiency. The ratio was high for the weakly silenced clones (Fig. 11B). The ratio for the most weakly silenced sub-clone (#5) was 2.19, while that for the most strongly silenced clone (#1) was 0.46.



### *Effect of TSA for elongation step of the aadA IR transcription*

I investigated the effect of TSA treatment on transcription elongation, i. e., ICP/CP-ratio was analyzed. To distinguish the incomplete *aadA* IR transcripts (ICP) from the complete *aadA* IR transcripts (CP) strictly, I used 280 nt *aadA* N-terminal sense-probe, which hybridizes to the 3'-end of the second-half *aadA* IR transcript, and 200 nt *aadA* C-terminal antisense-probe, which hybridizes to the end of the first-half of the *aadA* IR transcript. Therefore, I could detect nearly complete transcript by the N-terminal sense-*aadA* probe, while the C-terminal antisense-*aadA* probe was used to detect both incomplete and complete ones. Northern analysis indicated that 90 minutes TSA treatment had prominent effect to enhance complete transcription both for the weakly and strongly silenced sub-clones. The ICP/CP-ratio decreased to about one-third of the before treatment for both types of the sub-clones (Fig. 12). Unexpectedly, the amount of *aadA* mRNA, which assay was intended to be one of TSA treatment insensitive genes, in 19-P(1030) decreased to under detectable level by TSA treatment, however I had no reasonable interpretation for this phenomena (Fig. 12).

### **Discussion**

It has been reported that growth speed of a colony and the level of the spectinomycin resistance show positive relationship on appropriate concentration of spc/TAP-plates (Cerutti H *et al.*, 1997 (a)). Since RNAi-37 formed variable sizes of colonies on spectinomycin plates even after recurrent single colony isolations, this phenomenon was conceivable that RNAi efficiency was fluctuating among colonies through somatic cell divisions. On the other hand, the average RNAi efficiency of the population sifted to be lower, when RNAi-37 was subjected to succeeding cell culture in TAP medium for a very long period of time. Most probably, this is due to the increased number of cells that transcribe the *aadA* IR very limitedly. This is most probably related with the energy consumption. Cells of weaker RNAi response probably grow a bit faster than those with strong RNAi response, because of the lesser energy consumption in the weaker RNAi cells. Therefore, under the condition that RNAi has no advantage, such cells will increase the ratio in a population (Rohr J *et al.*, 2004).

Substantial amount of hairpin RNA was constantly detected from RNAi-37 by Northern-blot analyses (Fig. 2B, 4B), irrespective of the RNAi efficiency in the cells. I suppose that large portion of *aadA* IR transcript was retained in the nucleus where Dicer activity is probably negligible, and small parts of the accumulates are exported to cytoplasm little by little. The above scenario was supported by that increase of siRNA

had large delay to that of hairpin RNA, and it lasted for a long time (Fig. 10).

Poly-adenylation signal is encoded at the 3'-UTR end of the inverted repeat DNA, which is originally located in the *C. reinhardtii RbcS2* gene. However, I failed to recover the hairpin RNA using poly-T RNA conjugated beads even from total RNA (Fig. 13). Above suggests that our hairpin RNA carries no poly A tail, and therefore it is not efficiently incorporated into the mRNA transportation system.

Obvious reverse correlation was detected between the accumulated amount of hairpin RNA and the frequency of \*CpG in the hairpin RNA coding IR region. For various kinds of eukaryotes, it has been reported that \*CpG leads heterochromatin formation *via* various proteins that specifically bind to \*CpG. Moreover, accumulated \*CpG has potential to recruit diverse kinds of histone N-terminal modulators. Histone deacetylase is one of such enzymes.

It have been reported that in *Neurospora crassa* \*CpG in the coding region hampers the elongation step of the Pol II RNA polymerase (Rountree MR and Selker EU, 1997), and also in the case of mammals \*CpG positioned in the down stream region of promoters impedes the elongation step through heterochromatin formation (Lorincz MC *et al.*, 2004). Our observations described below in this study also support the formation of heterochromatin that was induced by \*CpG at the *aadA* IR region: (i) there

was a correlation between the frequency of \*CpG in the IR and the amount of 3'-truncated hairpin RNA, and (ii) TSA treatment reduced the incomplete transcripts.

Distribution of \*CpG showed clear gradient, which was richer in the region close to the promoter region than the middle part. This bias was clear irrespective of the efficiency of RNAi (Fig. 8). Considering that various 3'-truncated *aadA* IR transcripts were accumulated, \*CpG frequency bias might be correlated with how frequently the region was transcribed.

Elongation step of the transcription is significantly affected by various kinds of histone modifications besides deacetylation (Ng HH *et al.*, 2003; Hampsey M and Reinberg D, 2003; Krogan NJ *et al.*, 2003; Kizer KO *et al.*, 2005). Therefore, TSA treatment might have secondary effects on various histone modifications besides deacetylation. Altered histone modifications might enhance the full transcription of the hairpin RNA.

It is noteworthy, TSA treatment not only increased the ratio of completely transcribed *aadA* IR, but also prominently increased the total amount of the IR transcripts at once (Fig. 10). This is most probably due to the enhanced initiation of the transcription step, which is most likely a secondary effect of the improved elongation step. On the other hand, the amount of accumulated *ble* mRNA increased only slightly

by the same treatment (Fig. 11C).

Fluctuated spectinomycin resistance through somatic cell divisions is most probably caused by the incomplete maintenance of \*CpG through cell division, which leads to the different states of the IR region, i. e., whether the region is heterochromatin or euchromatin. These two chromatin states seem to be shuffling frequently in response to the frequency of \*CpG in *C. reinhardtii*.

In *A. thaliana*, siRNA directed DNA methylation occurs even when the target DNA region is silent for the transcription (Chan *et al.*, 2005; Herr *et al.*, 2005; Onodera *et al.*, 2005; Kanno *et al.*, 2005 (a), (b)). While the *aadA* gene has no obvious methylation even in RNAi induced cells of which target is *aadA* mRNA. Therefore, most probably *C. reinhardtii* has no system for siRNA directed DNA methylation. This is reinforced by that no evident genes for RNA dependent DNA methylase are coded in *C. reinhardtii* genome (Gendrel AV and Colot V. 2005). 5-methylcytosine reaches 30 % in the *A. thaliana* genome, while it is limited to 0.7% and 1.1% for *C. reinhardtii* and *Volvox carteri*, respectively. It is an open question how such a limited amount of \*C is operating in these green algae to regulate the gene expression. On transposons in *C. reinhardtii*, no prominent methylation was detected for a retro-transposon *TOCI* and a DNA transposon *Gulliver*, while another retro-transposon of long terminal repeat *REMI*

contains a detectable amount of <sup>14</sup>C (Jeong Br BR *et al.*, 2002; Zhang C *et al.*, 2002; Perez-Alegre M *et al.*, 2005).

I observed the increase of *TOC1* mRNA in response to the TSA treatment (Fig. 14). Moreover, the increment curve after TSA treatment was very similar to that for the hairpin RNA. It has been reported that transcription of *TOC1* is repressed by heterochromatin formation for which Mut11p, subunit of H3K4 methyltransferase complexes, and a novel serine/threonine kinase were participated (Jeong Br BR *et al.*, 2002; van Dijk K *et al.*, 2005). Therefore, these enzymes might be also participated to repress the IR DNA construct used in this study.

Introduced DNA fragment is often making a cluster in genome, probably because ligation of the introduced DNA fragments occurs in cytosol before integration into a genome (Kindle KL *et al.*, 1989; Mayfield SP, and Kindle KL, 1990). Such cluster is composed of inverted and direct repeat of the introduced DNA fragments, where various levels of <sup>14</sup>CpG accumulation in the region has been reported (Cerutti H *et al.*, 1997 (b); Babinger P *et al.*, 2001). Very recently, Casas-Mollano et al. (2007) showed that *SET3* (encoding histone H3 monomethyltransferase on lysine 9 (H3K9)) is required for silencing of tandemly repeated *aadA* genes in *C. reinhardtii* by RNAi-mediated suppression of this gene. Therefore, it is intriguing whether monomethyl H3K9 is also

acting as an epigenetic mark of repression for single inverted repeat constructs, such as a silencer construct used in this study, because RNAi-mediated repression of *SET3* did not release the silencing of single copy transgene or dispersed transposable elements, such as *TOC 1* retrotransposon (Casas-Mollano et al., 2007).

It is empirically known that silencer DNA construct composed of an intron containing genomic sequence and the complementary sequence lacking the intron part lasts longer than silencer construct composed of complete IR DNA sequence (Schroda M, 2006). Two mutually not exclusive reasons are possible for this: (i) incomplete IR silencer construct is more stable than complete IR silencer in genome, and (ii) incomplete IR sequence induces *de novo* C-methylation less frequently.

I showed on average about 20 % of the CpG sites in the promoterless IR construct were methylated. This frequency is much less than that is observed for the promoter bearing IR silencer (Ca. 50 %). This indicates that probably transcription improves the efficiency of *de novo* methylation or maintenance of the CpG, or transcription enhances both of them at once.

Using IR DNA construct that is driven by an inducible promoter, Koblenz and Lehtreck (2005) showed that deterioration of silencing effect occurs concomitantly with transcription. Strong RNAi was observed when transformants were shifted to

inducible conditions even after long period of succeeding culture, so far as it had been cultured under non-inducible conditions. This also suggests that transcription enhances the \*CG in an IR structure.

It is known that transcription urges remodeling of nucleosome in cooperation with remodeling factors. While maintenance of \*CpG also requires remodeling factors such as *DDMI* besides methyltransferase (Jeddeloh JA *et al.*, 1999). Therefore, most probably transcription factors are implicated in the maintenance of \*CpG in IR structure as well as the \*C recognition proteins such as MeCP2, MBDs (Nan X *et al.*, 1998; Jones PL *et al.*, 1998). The above explains reasonably why heavy accumulation of \*CpG was observed for the transcribable *aadA* IR. Moreover, in *A. thaliana*, nascent transcript of IR DNA interacts with their coding regions to induce a dimethyl modification to histone H3 on lysine 9 ( H3K9me2) in cooperation with SUV4/6 (Ebbs ML *et al.*, 2005; Ebbs ML, and Bender J, 2006), while histone H3K9me2 modification is closely related with \*CpG. Similar mechanisms might be working in *C. reinhardtii* to enhance \*CpG accumulation at the *aadA* IR construct carrying a promoter. Suggesting the wide distribution of DNA structure dependent cytosine methylation in plants, IR DNA sequence dependent C-methylation also has been reported in *A. thaliana* (Melquist S, and Bender J, 2003). Close analysis of IR DNA dependent methylation in *A. thaliana*



showed that \*CpG DNA methylation is a result of *de novo* C-methylation and the maintenance for \*CpG among \*CpN through DNA replications. Artificial gene that contains \*CpG was introduced into *A. thaliana* genome and its maintenance was chased. This experiment showed that the successful \*CpG maintenance ratio was 96% per DNA replication (Bird A. 2002). In the case of *C. reinhardtii*, the number of \*CpG sites in the IR seems fluctuating through cell divisions more vigorously than in *A. thaliana*. Therefore, \*CpG fluctuation in *C. reinhardtii* is probably owing to that *de novo* methylation occurs seldom and maintenance of \*CpG is not so faithful as in *A. thaliana*. Fluctuation of \*CpG frequency through cell divisions might be not limited to *C. reinhardtii*. Eye color variegation in *D. melanogaster* is one of the typical cases (Reuter G and Spierer P, 1992) in which such fluctuation was observed in a multicellular organism.

**Adams RL** (1990). DNA methylation. The effect of minor bases on DNA-protein interactions. *Biochem J.* **265**, 309-320.

**Babinger P, Kobl I, Mages W, and Schmitt R** (2001). A link between DNA methylation and epigenetic silencing in transgenic *Volvox carteri*. *Nucleic Acids Res.* **29**, 1261-1271.

**Bird, A** (2002). DNA methylation patterns and epigenetic memory. *Genes Dev.* **16**, 6-21.

**Boynton JE, Gillham NW, Harris EH, Hosler JP, Johnson AM, Jones AR, Randolph-Anderson BL, Robertson D, Klein TM, Shark KB, et al.** (1988) Chloroplast transformation in *Chlamydomonas* with high velocity microprojectiles. *Science.* **240**, 1534-1538.

**Casas-Mollano JA, van Dijk K, Eisenhart J, and Cerutti H** (2007) SET3p monomethylates histone H3 on lysine 9 and is required for the silencing of tandemly repeated transgenes in *Chlamydomonas*. *Nucleic Acids Res.* **35**, 939-950.

**Cerutti H, Johnson AM, Gillham NW, and Boynton JE** (1997) (a) A eubacterial gene conferring spectinomycin resistance on *Chlamydomonas reinhardtii*: integration into the nuclear genome and gene expression. *Genetics.* **145**, 97-110.

**Cerutti H, Johnson AM, Gillham NW, and Boynton JE** (1997) (b) Epigenetic

- silencing of a foreign gene in nuclear transformants of *Chlamydomonas*. Plant Cell. **9**, 925-945.
- Cerutti H** (2003) RNA interference: traveling in the cell and gaining functions? Trends Genet. **19**, 39-46.
- Chan SW, Henderson IR, and Jacobsen SE** (2005) Gardening the genome: DNA methylation in *Arabidopsis thaliana*. Nat Rev Genet. **6**, 351-360.
- Gendrel AV and Colot V** (2005) *Arabidopsis* epigenetics: when RNA meets chromatin. Curr Opin Plant Biol. **8**, 142-147.
- Gonzalez-Ballester D, de Montaigu A, Galvan A, and Fernandez E** (2005) Restriction enzyme site-directed amplification PCR: a tool to identify regions flanking a marker DNA. Anal Biochem. **340**, 330-335.
- Grossman AR, Harris EE, Hauser C, Lefebvre PA, Martinez D, Rokhsar D, Shrager J, Silflow CD, Stern D, Vallon O, and Zhang Z** (2003) *Chlamydomonas reinhardtii* at the crossroads of genomics. Eukaryot Cell. **2**, 1137-50
- Jeddeloh JA, Stokes TL, and Richards EJ** (1999) Maintenance of genomic methylation requires a *SWI2/SNF2*-like protein. Nat Genet. **22**, 94-97.
- Jeong Br BR, Wu-Scharf D, Zhang C, and Cerutti H** (2002) Suppressors of transcriptional transgenic silencing in *Chlamydomonas* are sensitive to

DNA-damaging agents and reactivate transposable elements. Proc Natl Acad Sci U S A. **99**, 1076-1081.

**Jones PL, Veenstra GJ, Wade PA, Vermaak D, Kass SU, Landsberger N, Strouboulis J, and Wolffe AP** (1998) Methylated DNA and *MeCP2* recruit histone deacetylase to repress transcription. Nat Genet. **19**, 187-191.

**Hampsey M, and Reinberg D** (2003) Tails of intrigue: phosphorylation of RNA polymerase II mediates histone methylation. Cell. **113**, 429-32.

**Hattman S, Kenny C, Berger L, and Pratt K** (1978) Comparative study of DNA methylation in three unicellular eukaryotes. J Bacteriol. **135**, 1156-1157.

**Herr AJ, Jensen MB, Dalmay T, and Baulcombe DC** (2005) RNA polymerase IV directs silencing of endogenous DNA. Science. **308**, 118-120.

**Kanno T, Huettel B, Mette MF, Aufsatz W, Jaligot E, Daxinger L, Kreil DP, Matzke M, and Matzke AJ** (2005) (a) Atypical RNA polymerase subunits required for RNA-directed DNA methylation. Nat Genet. **37**, 761-765.

**Kanno T, Aufsatz W, Jaligot E, Mette MF, Matzke M, and Matzke AJ** (2005) (b) A *SNF2*-like protein facilitates dynamic control of DNA methylation. EMBO Rep. **6**, 649-55.

- Kindle KL, Schnell RA, Fernandez E, and Lefebvre PA** (1989) Stable nuclear transformation of *Chlamydomonas* using the *Chlamydomonas* gene for nitrate reductase. *J Cell Biol.* **109**, 2589-601.
- Kizer KO, Phatnani HP, Shibata Y, Hall H, Greenleaf AL, and Strahl BD** (2005) A novel domain in *Set2* mediates RNA polymerase II interaction and couples histone H3 K36 methylation with transcript elongation. *Mol Cell Biol.* **25**, 3305-16.
- Koblenz B, and Lehtreack KF** (2005) The *NIT1* promoter allows inducible and reversible silencing of centrin in *Chlamydomonas reinhardtii*. *Eukaryot Cell.* **4**, 1959-1962.
- Krogan NJ, Kim M, Tong A, Golshani A, Cagney G, Canadien V, Richards DP, Beattie BK, Emili A, Boone C, Shilatifard A, Buratowski S, and Greenblatt J** (2003) Methylation of histone H3 by *Set2* in *Saccharomyces cerevisiae* is linked to transcriptional elongation by RNA polymerase II. *Mol Cell Biol.* **23**, 4207-18.
- Leon-Banares R, Gonzalez-Ballester D, Galvan A, and Fernandez E** (2004) Transgenic microalgae as green cell-factories. *Trends Biotechnol.* **22**, 45-52.
- Liu, L., Wylie, R., Hansen, N., Andrews, L.G., and Tollefsbol, T.O** (2004) Profiling DNA methylation by bisulfite genomic sequencing; problems and solutions. *Methods in Molecular Biology.* **287**, 169-179.

**Loidl P** (2004) A plant dialect of the histone language. *Trends Plant Sci.* **9**, 84-90.

**Lorincz MC, Dickerson DR, Schmitt M, and Groudine M** (2004) Intragenic DNA methylation alters chromatin structure and elongation efficiency in mammalian cells. *Nat Struct Mol Biol.* **11**, 1068-75.

**Lumbreras V, Stivens DR, and Purton S** (1998) Efficient foreign gene expression in *Chlamydomonas reinhardtii* mediated by an endogenous intron. *Plant J* **14**, 441-447.

**Mayfield SP, and Kindle KL** (1990) Stable nuclear transformation of *Chlamydomonas reinhardtii* by using a *C. reinhardtii* gene as the selectable marker. *Proc Natl Acad Sci U S A.* **87**, 2087-2091.

**Melquist S, and Bender J** (2003) Transcription from an upstream promoter controls methylation signaling from an inverted repeat of endogenous genes in *Arabidopsis*. *Gene Dev.* **17**, 2036-2047.

**Ebbs ML, Bartee L, and Bender J** (2005) H3 lysine 9 methylation is maintained on a transcribed inverted repeat by combined action of *SUVH6* and *SUVH4* methyltransferases. *Mol Cell Biol.* **25**, 10507-10515.

**Ebbs ML, and Bender J** (2006) Locus-specific control of DNA methylation by the *Arabidopsis SUVH5* histone methyltransferase. *Plant Cell.* **18**, 1166-1176.

**Nan X, Ng HH, Johnson CA, Laherty CD, Turner BM, Eisenman RN, and Bird A**

- (1998) Transcriptional repression by the methyl-CpG-binding protein *MeCP2* involves a histone deacetylase complex. *Nature*. **393**, 386-389.
- Ng HH, Robert F, Young RA, and Struhl K** (2003) Targeted recruitment of *Set1* histone methylase by elongating Pol II provides a localized mark and memory of recent transcriptional activity. *Mol Cell*. **11**, 709-19.
- Onodera Y, Haag JR, Ream T, Nunes PC, Pontes O, and Pikaard CS** (2005) Plant nuclear RNA polymerase IV mediates siRNA and DNA methylation-dependent heterochromatin formation. *Cell*. **120**, 613-622.
- Perez-Alegre M, Dubus A, and Fernandez E** (2005) *REMI*, a new type of long terminal repeat retrotransposon in *Chlamydomonas reinhardtii*. *Mol Cell Biol*. **25**, 10628-10638.
- Randolph-Anderson BL, Boynton JE, Gillham NW, Harris EH, Johnson AM, Dorthu MP, and Matagne RF** (1993) Further characterization of the respiratory deficient *dum-1* mutation of *Chlamydomonas reinhardtii* and its use as a recipient for mitochondrial transformation. *Mol Gen Genet*. **236**, 235-44.
- Reuter G, and Spierer P** (1992) Position effect variegation and chromatin proteins. *Bioessays*. **14**, 605-12.

**Rohr J, Sarkar N, Balenger S, Jeong BR, and Cerutti H** (2004) Tandem inverted repeat system for selection of effective transgenic RNAi strains in *Chlamydomonas*. Plant J. **40**, 611-21.

**Rountree MR and Selker EU** (1997) DNA methylation inhibits elongation but not initiation of transcription in *Neurospora crassa*. Genes Dev. **11**, 2383-2395.

**Sambrook, J., and Russell, D. W** (2001). Molecular Cloning: A laboratory Manual. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).

**Schroda M, Beck CF, and Vallon O** (2002) Sequence elements within an *HSP70* promoter counteract transcriptional transgene silencing in *Chlamydomonas*. Plant J. **31**, 445-455.

**Schroda M** (2006) RNA silencing in *Chlamydomonas*: mechanisms and tools. Curr Genet. **49**, 69-84.

**Tariq M and Paszkowski J** (2004) DNA and histone methylation in plants. Trends Genet. **20**, 244-251.

**van Dijk K, Marley KE, Jeong BR, Xu J, Hesson J, Cerny RL, Waterborg JH, and Cerutti H** (2005) Monomethyl histone H3 lysine 4 as an epigenetic mark for silenced euchromatin in *Chlamydomonas*. Plant Cell. **17**, 2439-2453.

**Watanabe KI, and Ohama T** (2001) Regular spliceosomal introns are invasive in



*Chlamydomonas reinhardtii*: 15 introns in the recently relocated mitochondrial *cox2* and *cox3* genes. J Mol Evol. **53**, 333-339.

**Yamasaki T, Kurokawa S, Watanabe KI, Ikuta K, and Ohama T (2005)** Shared molecular characteristics of successfully transformed mitochondrial genomes in *Chlamydomonas reinhardtii*. Plant Mol Biol. **58**, 515-527.

**Zhang C, Wu-Scharf D, Jeong BR, and Cerutti H (2002)** A WD40-repeat containing protein, similar to a fungal co-repressor, is required for transcriptional gene silencing in *Chlamydomonas*. Plant J. **31**, 25-36.

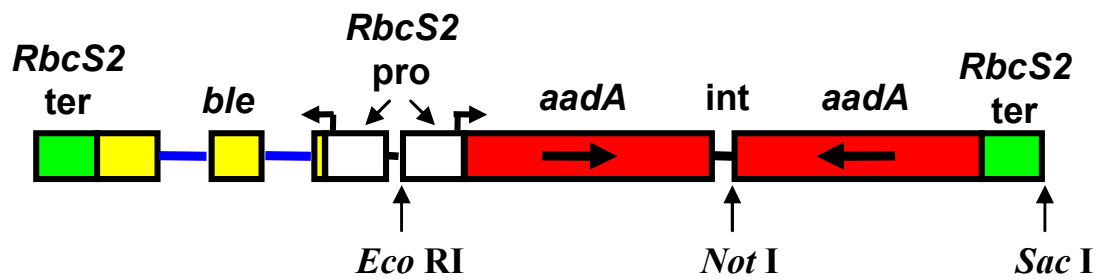


Figure 1. Schematically shown silencer DNA construct and a marker gene.

Restriction enzymes and their sites utilized to make this construct are indicated with arrows. 770 bp coding region for *aadA* gene (shown in red) with deletion of the first 15 bases was arranged to make an inverted repeat. 79 bp *cox2-no2*-intronic DNA sequence was located in the middle of the inverted repeat. A *ble* gene (shown in yellow) was used as a marker gene of transformation. This gene is carrying two *RbcS2* first introns (shown by a blue bar) where enhancer activity is enclosed. To regulate transcriptional activity of this silencer construct and *ble* marker gene *C. reinhardtii* *RbcS2* promoter (shown in white) and *RbcS2* terminator (shown in green) were used. Silencer DNA construct and a marker gene were arranged head to head to have the opposite direction for their transcription.

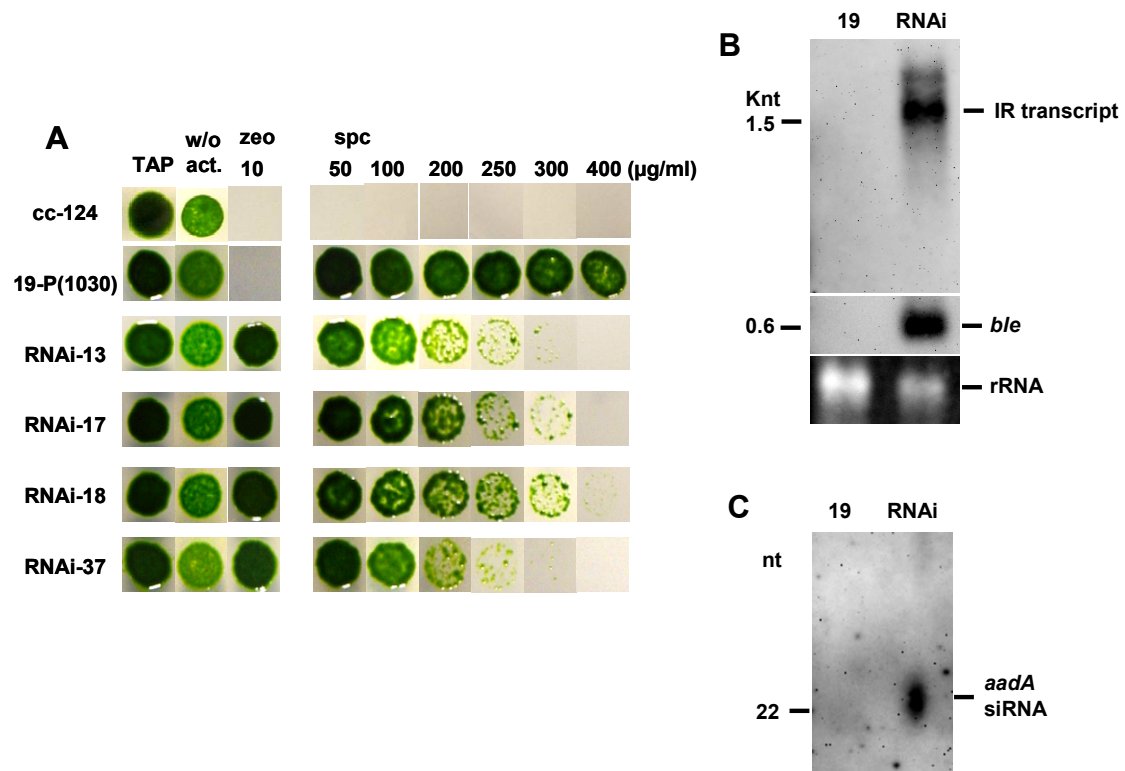


Figure 2. Analysis of RNAi induced cell features.

(A) Results of spotting test for silencer DNA introduced transformants.

cc-124:wild type; 19-P(1030):stably *aadA* mRNA expressing transformants of cc-124;

RNAi-13, -17, -18, and -37:transformants of 19-P(1030) by introduction of a silencer

DNA plasmid linealized by *Kpn* I. TAP:Tris-Acetate-Phosphate medium; w/o act.:TAP

medium without acetate; zeo:zeocin; spc: spectinomycin. See Materials and Methods

for the procedures of the spotting test.

(B) Detection of *aadA* IR transcript by Northern blot analysis. Hairpin RNA

transcribed from the silencer DNA construct was detected by DIG-labeled sense-*aadA*

RNA probe (see Materials and Methods). Analysis of the same blot with a *ble* probe (see Materials and Methods) was carried out. About 10 µg of total RNA was loaded per lane. Ethidium bromide staining of the agarose gel was carried out to check the amount and conditions of the loaded RNA.

(C) Detection of siRNA. Small RNA fraction prepared from 30 µg of total RNA was loaded to 8 M urea containing 15 % acrylamide gel. Detection was carried out by sense-*aadA* RNA probe.

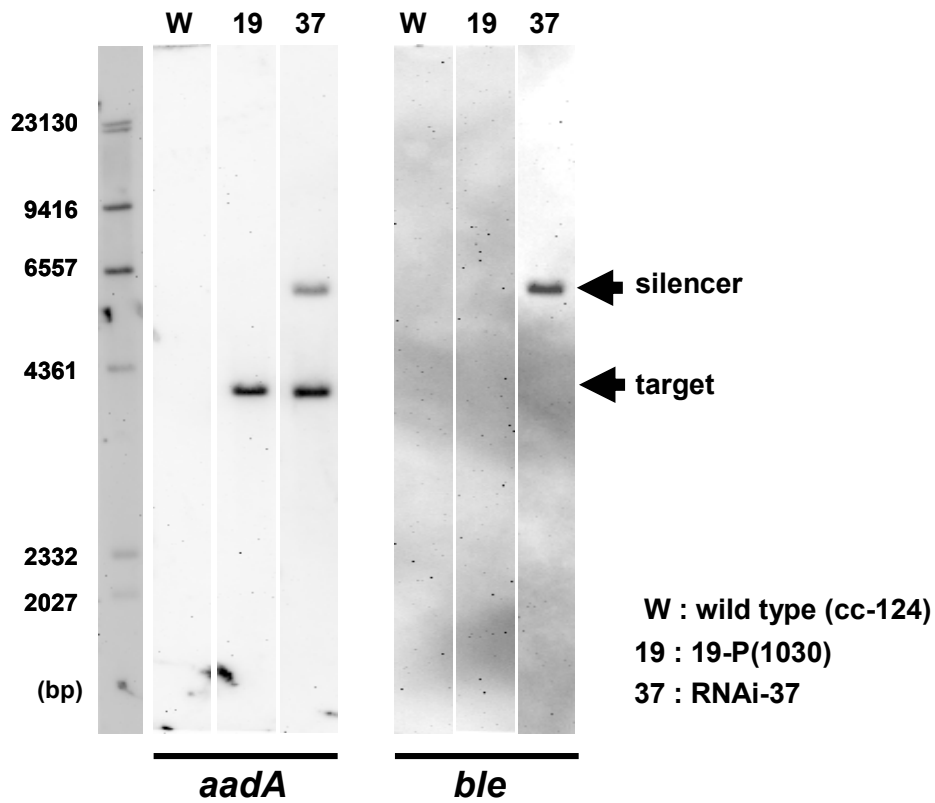


Figure 3. Southern hybridization analyses of RNAi-37.

Detection of *aadA* and *ble* signals. Total genomic DNA of cc-124 (W), 19-P(1030) (19), and RNAi-37 (37) were completely digested with *Sac* I. Signals were detected with *aadA* N-terminal side probe (left panel) and *ble* (right panel) probe.

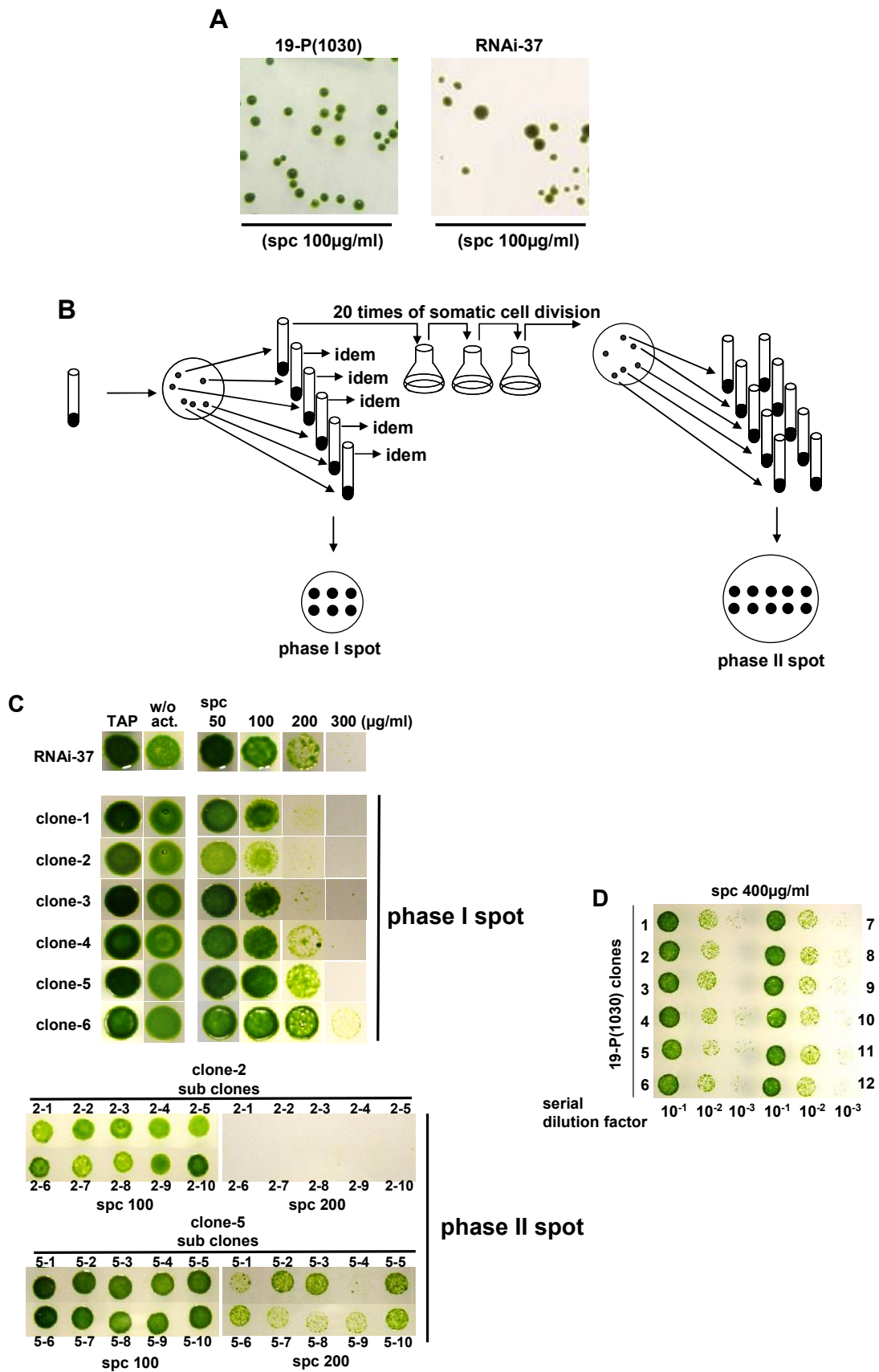


Figure 4. Spotting test procedures for fluctuating spectinomycin resistance.

(A) Growth of RNAi-37 on spectinomycin containing agar plate. After succeeding culture of RNAi-37, small portion of the culture was spread on 100 µg/ml spectinomycin containing agar plate, and incubated for 10 days. Same operation was carried out for 19-P(1030) that is expressing *aadA* mRNA stably.

(B) Flow chart of spotting test. After more than 100 times of somatic cell divisions, RNAi-37 was spread on TAP plate, then 6 colonies were isolated and their spectinomycin resistance was analyzed using various spectinomycin plates (Phase I spotting).

Each RNAi-37 clone was subjected to additional cell divisions (more than 20 times), then spread on TAP plates. 10 randomly chosen sub-clones for each one of the six RNAi-37 clones (totally 60 sub-clones) were spotted on various spectinomycin plates (Phase II spotting).

(C) Results of spotting test for RNAi-37 sub-clones.

(D) Result of spotting test for 19-P(1030).

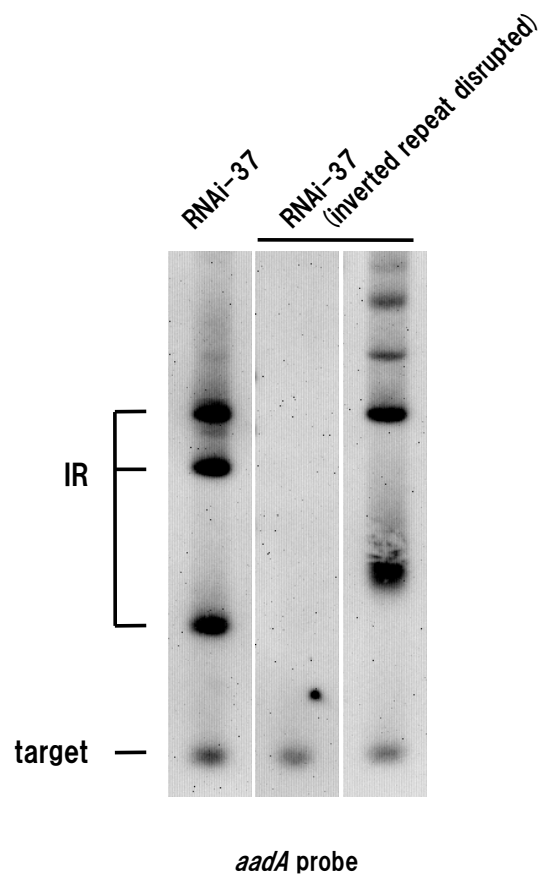


Figure 5. Southern hybridization analyses of RNAi-37 clones that show fully recovered spectinomycin resistance.

Total genomic DNA was prepared from spectinomycin resistant RNAi-37 clones that grow on TAP/spc400 as faster as 19-P(1030), and also strongly silenced RNAi-37 clones. They were completely digested with *Sac* I. Hybridization was carried out using full length of *aadA* DNA probe. Intact silencer DNA construct in RNAi-37 exhibited three bands. However, fully spectinomycin resistant clones showed different pattern of signals, indicating rearrangement happened in the silencer construct.



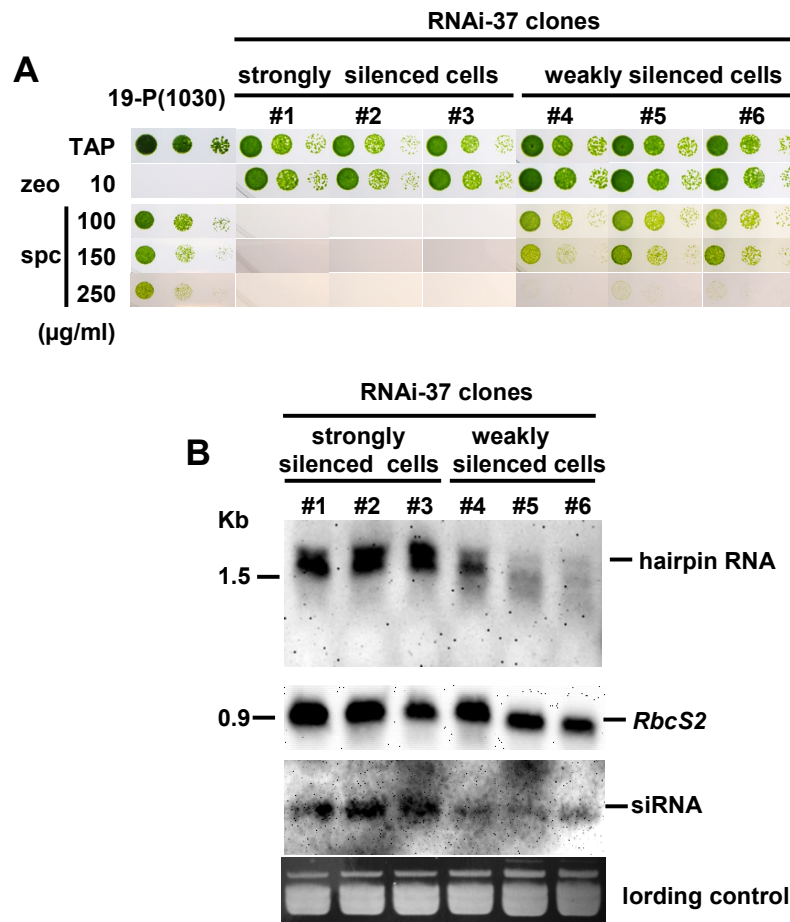


Figure 6. Comparative analyses of RNAi efficiency and the amount of siRNA and hairpin RNA.

(A) Evaluation of spectinomycin resistance for 6 RNAi-37 clones. Three strongly silenced sub-clones (#1-#3) and weakly silenced sub-clones (#4-#6) were spotted on various kinds of plates after serial dilutions of  $10^{-1}$ ,  $10^{-2}$ , and  $10^{-3}$ .

(B) Northern blot analysis for hairpin RNA and siRNA. Sense-*aadA* RNA probe was used to detect hairpin RNA and also siRNA. Total RNA was loaded to 1 %

agarose/formaldehyde gel to detect hairpin RNA, while silencer was detected from small RNA fraction after resolving it using 8 M urea containing 15 % acrylamide gel. Ethidium bromide staining and detection of *RbcS2* mRNA were carried out to confirm there is no describable difference for the loaded amount of the RNA among the lanes. Hybridization using *RbcS2* RNA probe was carried out lastly, because complete stripping of it was difficult.

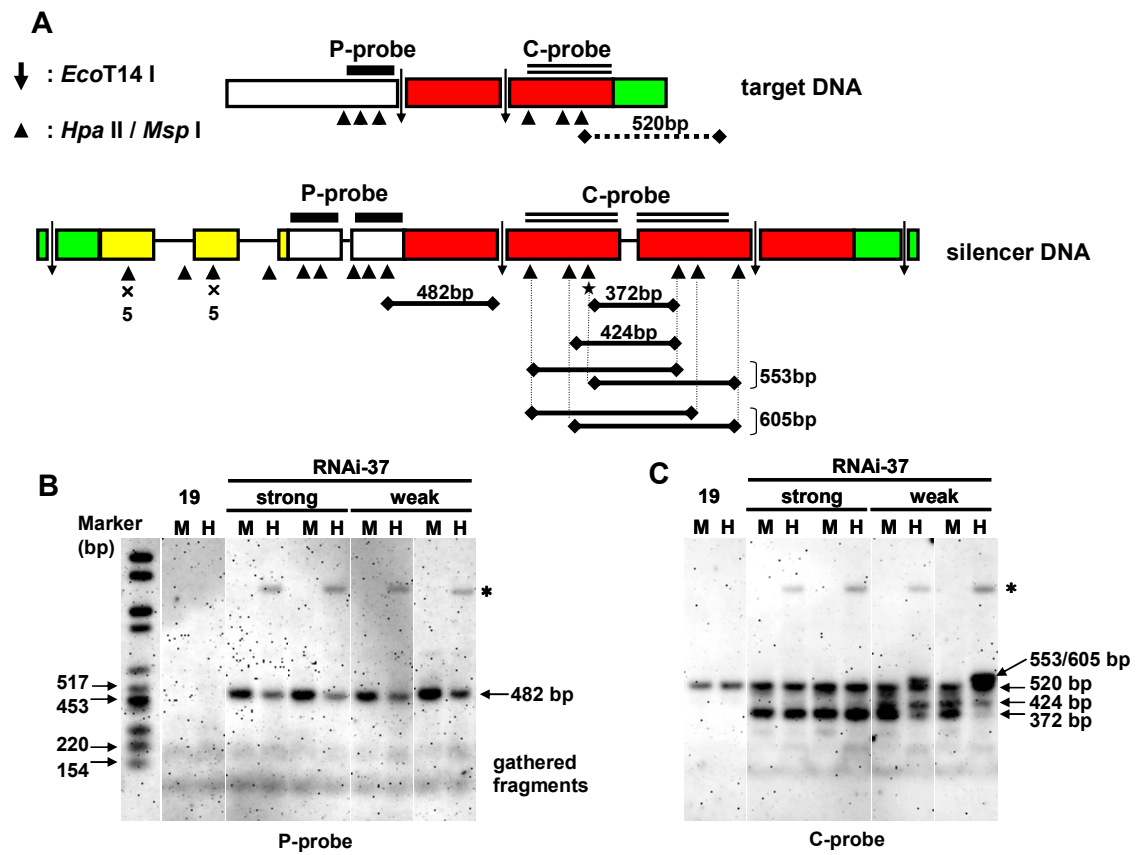


Figure 7. Methyated cytosine analysis using restriction enzymes.

(A) Schematic restriction maps for marker *ble* gene and silencer inverted repeat construct. The positions where P- and C-probe hybridize are shown by horizontal bars. Horizontal lines were corresponding to the detected signals shown in (B) and (C).

(B) Result of hybridization by P-probe. 19: DNA prepared from 19-P(1030); weak: genome DNA prepared from weakly silenced RNAi-37 clones were loaded; strong: genome DNA from strongly silencing clones were used. \*: unknown bands. Gathered fragments: unresolved small DNA fragments because of insufficient agarose

gel concentration.

(C) Result of hybridization by C-probe.

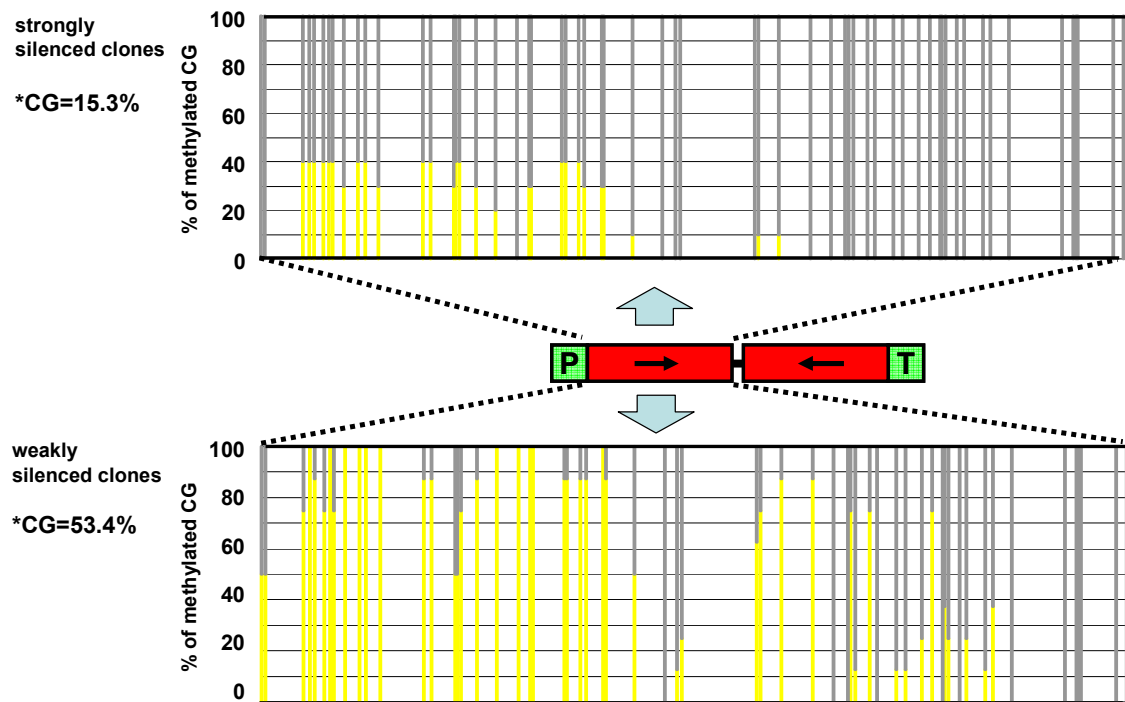


Figure 8. Analyses of methylated cytosine in the inverted repeat region of the silencer DNA construct.

Position and frequency of methylated cytosine were analyzed by the sodium bisulfate genomic sequencing method for front-half of the inverted repeat. 10 PCR products raised from weakly silenced RNAi-37 sub-clones, and 8 PCR products from strongly silenced RNAi-37 sub-clones were sequenced. Appearance of methylated cytosine was limited to C in the CpG context. Frequency and relative position of methylated cytosine were shown by a yellow bar.

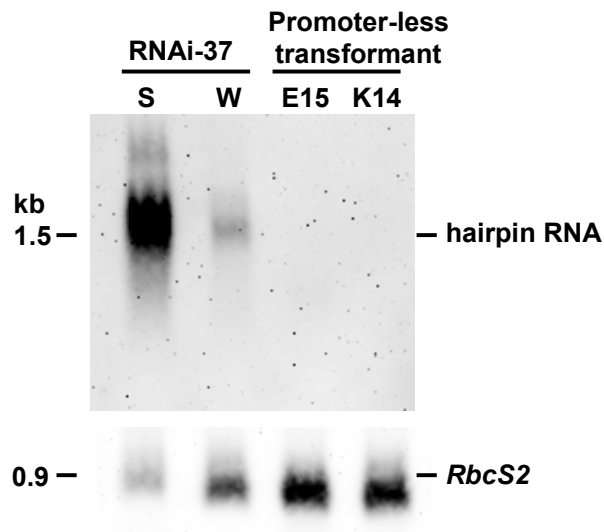


Figure 9. Northern blot analyses of promoter-less silencer construct integrated transformants.

Two transformants (E15 and K14) were generated by transformation with the remodeled silencer DNA construct, which has no *RbcS2* promoter and terminator regions. Northern blot analyses were carried out using total RNA prepared from E15 and K14 transformants, as well as intact silencer carrying RNAi-37 strain. Signal were detected with *aadA* N-terminal sense probe (upper panel) and *RbcS2* anti-sense probe (lower panel)

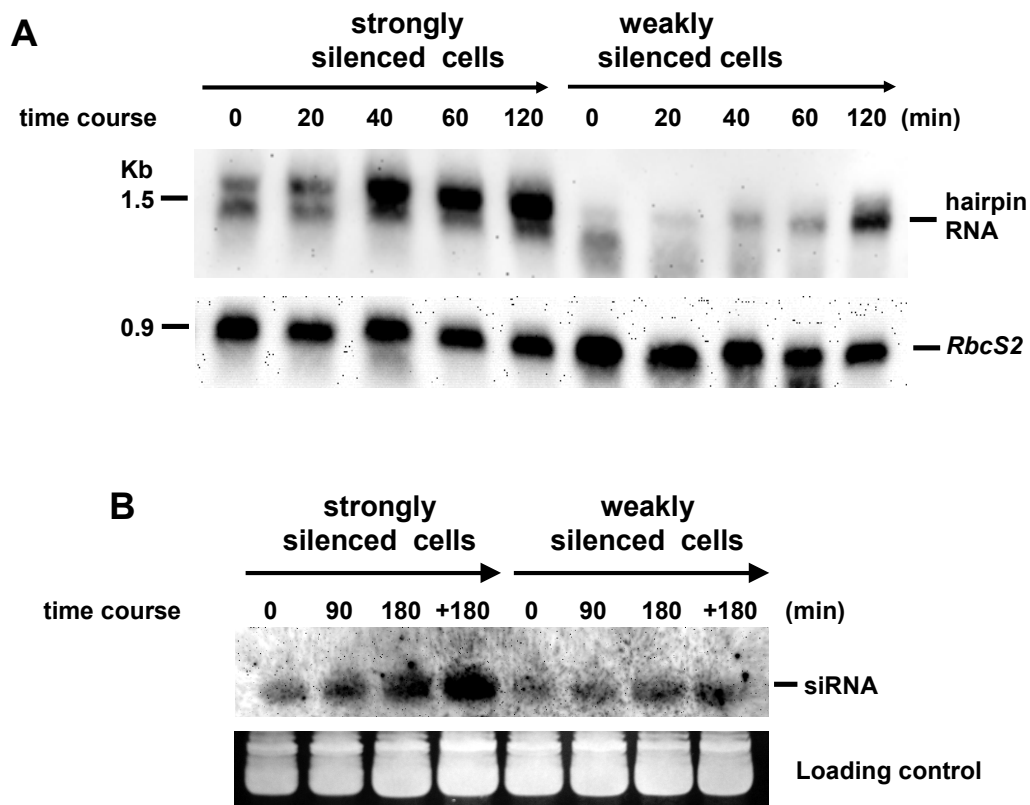


Figure 10. Time course change of *aadA* IR transcript by TSA treatment.

(A) Accumulated amount of hairpin RNA was analyzed by addition of TSA in the culture medium. Northern blot analyses were carried out using DIG-labeled sense-*aadA* RNA probe. Detection of *RbcS2* mRNA was also carried out to confirm that equal amount of RNA was loaded per lane.

(B) Time course change of siRNA by TSA treatment. Small RNA fraction was prepared from aliquots of the cell culture, which were taken at different times. DIG-labeled sense-*aadA* RNA probe was used to detect the siRNA. Ethidium bromide staining of RNA was carried out to confirm that equal amount of RNA was applied per

lane. +180: Cells were transferred to fresh TAP medium after 180 minutes of incubation in the TSA containing TAP medium, and then additional incubation was carried out for 180 minutes.



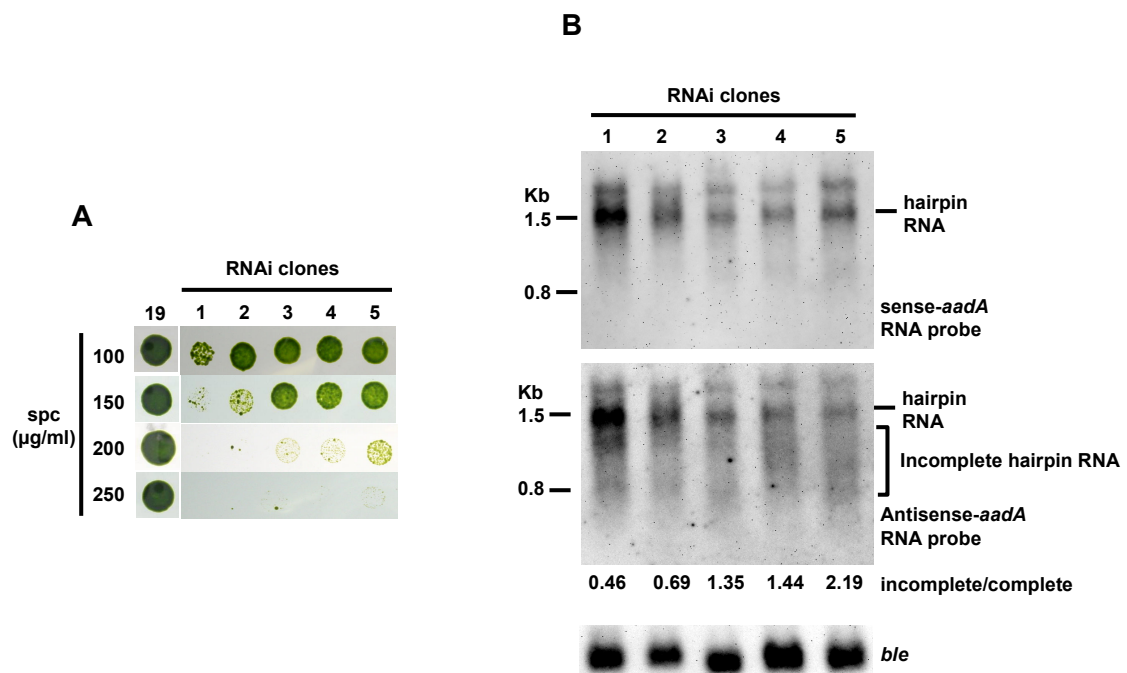


Figure 11. Analyses of silencer DNA transcripts.

(A) Five RNAi-37 clones, which show distinctive efficiency of silencing were chosen. Spotting test was carried out using various concentrations of spectinomycin plates. The smaller number of clones have higher efficiency of silencing.

19:19-P(1030).

(B) Detection of silencer DNA transcripts by different probes. N-terminal sense-*aadA* RNA probe hybridizes to almost completely transcribed products, while C-terminal antisense-*aadA* RNA probe hybridizes to even incomplete transcripts of the silencer DNA. Same membrane was re-used for these experiments. The ratio between the complete transcript and incomplete transcripts was calculated using NIH Image, and

the ratios were shown below the lanes. Hybridization signal for *ble* mRNA shows equal amount of total RNA was loaded per lane.

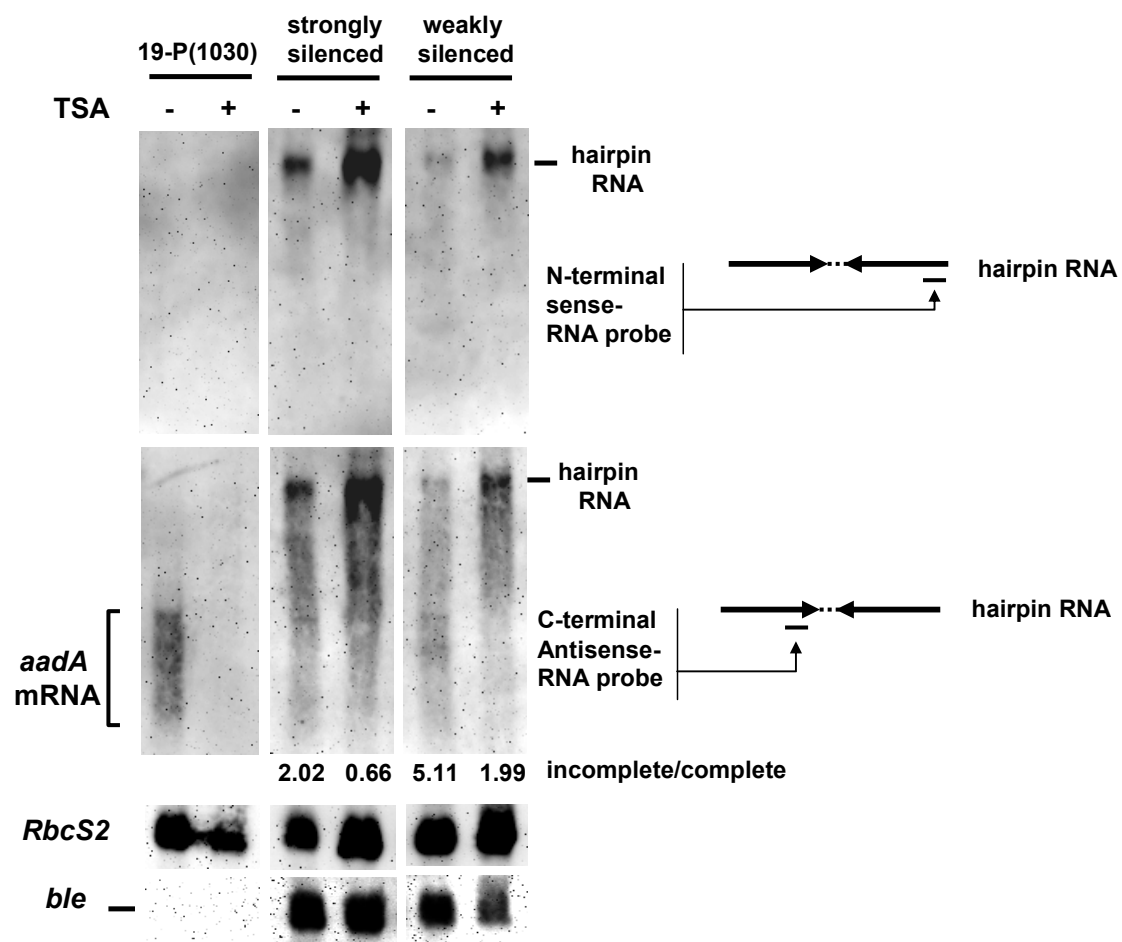


Figure 12. Effect of TSA treatment on silencer DNA transcripts.

Total RNA was prepared from the same amount of cell culture before and after addition of Trichostatin A (TSA) for 90 minutes. N-terminal sense-*aadA* RNA probe and C-terminal antisense-*aadA* RNA probe were used to strictly distinguish the complete transcript from the incomplete ones. The positions of the probe were also shown schematically.

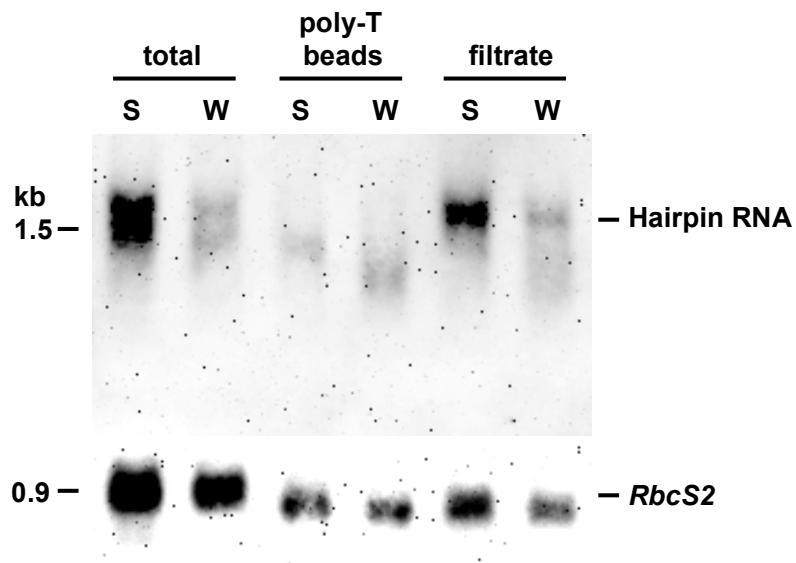


Figure 13. Northern blot analysis of using poly A<sup>+</sup> RNA prepared from strongly silenced and weakly silenced clones of RNAi-37.

Poly A<sup>+</sup> RNA was collected with Oligotex-dT60 (Super) (TaKaRa). Total RNA (total), poly A<sup>+</sup> RNA (poly T beads), and RNA in the filtrate (filtrate) were used for Northern blot analysis. Hairpin RNA was detected with *aadA* N-terminal sense probe (upper panel). *RbcS2* mRNA was detected with *RbcS2* anti-sense probe (lower panel).

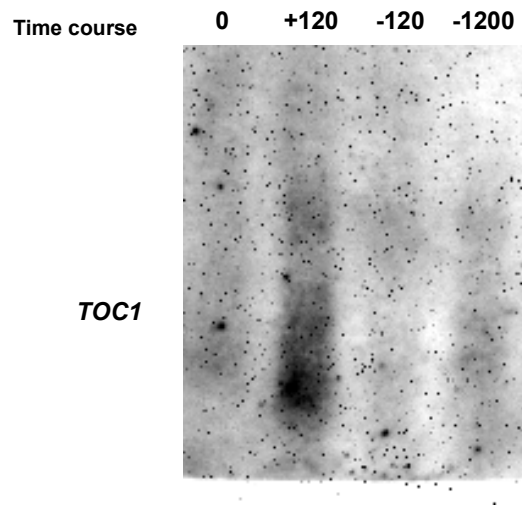


Figure 14. Northern blot analysis of *Chlamydomonas* transposon *TOC1*.

Total RNA was prepared from the TSA treated RNAi-37 cells. 0: before TSA treatment.

+120: incubation with TSA/TAP for 120 minutes. -120: incubation without TSA for 120

minutes after incubation with TAP/TSA medium for 120 minutes. -1200: incubation

without TSA for 1200minutes after incubation with TSA/TAP medium for 120 minutes.