TECHNICAL ADVANCE

Tandem inverted repeat system for selection of effective transgenic RNAi strains in *Chlamydomonas*

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Summary

RNA interference (RNAi), the double-stranded RNA (dsRNA) triggered post-transcriptional gene silencing, is becoming a powerful tool for reverse genetics studies. Stable RNAi, induced by the expression of inverted repeat (IR) transgenes, has been achieved in protozoa, algae, fungi, plants, and metazoans. However, the level of gene silencing is often quite variable, depending on the type of construct, transgene copy number, site of integration, and target gene. This is a hindrance in functional genomics studies, where it is desirable to suppress target genes reliably to analyze unknown phenotypes. Consequently, we explored strategies for direct selection of effective transgenic RNAi lines in *Chlamydomonas reinhardtii*. We initially attempted to suppress expression of the Rubisco small subunit multigene family by placing an IR, homologous to the conserved coding sequence, in the 3′UTR of a transgene conferring resistance to bleomycin. However, this approach was fairly inefficient at inducing RNAi as many strains displayed defective transgene integration, resulting in partial or complete deletion of the IR, or low levels of dsRNA expression, presumably due to transcriptional silencing of the integrated IR transgenes. To overcome these problems we designed a system consisting of tandem IR transgenes that consistently triggered co-silencing of a gene with a selectable RNAi-induced phenotype (encoding tryptophan synthase β subunit) and another gene of interest (encoding either Ku80, an RNA-binding protein, or a thioredoxin isoform). We anticipate that this approach will be useful for generating stable hypomorphic epi-mutants in high-throughput phenotypic screens.

Keywords: RNA interference, post-transcriptional gene silencing, inverted repeat transgene, reverse genetics, functional genomics.

Introduction

The genomes of several animal, fungal, and plant species have already been sequenced and that of the unicellular green alga *Chlamydomonas reinhardtii* is nearing completion (http://genome.jgi-psf.org). This has resulted in the identification of numerous genes that are functionally uncharacterized, posing a key challenge to our understanding of cellular behavior and organismal development. Reverse genetics approaches, such as insertional mutagenesis, tilling, and targeted gene disruption, have been successfully used to analyze gene function in eukaryotes (Greene *et al.*, 2003; Waterhouse and Helliwell, 2003). However, many of these strategies are laborious, requiring extensive screening

to identify specific gene mutations, and/or limited by the selectivity of mutational targeting. Furthermore, the direct manipulation of target genes by homologous recombination has proved difficult in many eukaryotes, including higher plants (Hanin and Paszkowski, 2003; Iida and Terada, 2004). As an alternative, RNA interference (RNAi) has recently emerged as a useful tool for functional genomic analyses (Dykxhoorn *et al.*, 2003; Gonczy *et al.*, 2000; Hope, 2001; Paddison and Hannon, 2002; Waterhouse and Helliwell, 2003). This approach exploits evolutionarily conserved cellular mechanisms involved in defense responses against viruses and transposons as well as in endogenous gene

regulation (Carrington and Ambros, 2003; Cerutti, 2003; Hannon, 2002; Matzke et al., 2001; Murchison and Hannon, 2004; Plasterk, 2002).

RNAi-related phenomena appear to be triggered by double-stranded RNA (dsRNA) and can lead to gene silencing via several pathways (Cerutti, 2003; Hannon, 2002; Matzke et al., 2001, 2004; Murchison and Hannon, 2004). However, experimentally induced RNAi is usually aimed at the degradation of specific target transcripts. According to the prevailing model, a ribonuclease III-like enzyme, Dicer, processes long dsRNAs into 21–26-nt small interfering RNAs (siRNAs). These small RNAs are then incorporated into a multiprotein complex, the RNA-induced silencing complex (RISC), that uses the siRNAs as guides to identify and cleave homologous mRNAs (Cerutti, 2003; Dykxhoorn et al., 2003; Hannon, 2002; Murchison and Hannon, 2004; Plasterk, 2002; Zamore, 2002). In some organisms, an amplification step, mediated by an RNA-directed RNA polymerase, is also required for efficient gene suppression (Cerutti, 2003; Hannon, 2002; Plasterk, 2002; Zamore, 2002).

RNAi can be induced by introduction of exogenously synthesized dsRNAs or siRNAs into cells, embryos, or whole organisms (Dykxhoorn et al., 2003; Klahre et al., 2002; Paddison and Hannon, 2002; Schweizer et al., 2000). However, the silencing effects of exogenous dsRNAs are generally transient and not heritable (Kennerdell and Carthew, 2000; Klahre et al., 2002; Misquitta and Paterson, 1999; Paddison and Hannon, 2002; Waterhouse and Helliwell, 2003). Moreover, genome-wide screens conducted by feeding *Caenorhabditis elegans* with bacteria expressing dsRNA have revealed significant variability between experiments and persistent levels of false negatives (i.e. target genes with known mutant phenotypes not showing RNAi-induced phenotypes) (Kamath et al., 2003; Simmer et al., 2003). Infiltration of cells with transgene-carrying *Agrobacterium tumefaciens* or infection with recombinant viral vectors have also been used for transient RNAi in plants (Gossele et al., 2002; Ratcliff et al., 2001; Robertson, 2004; Waterhouse and Helliwell, 2003). In the latter approach, termed virus-induced gene silencing (VIGS), modified viruses containing exogenous sequences can trigger RNAi against plant-encoded genes (Robertson, 2004).

In contrast to the strategies described above, RNAi triggered by genome integrated transgenes is heritable. Stable RNAi has been developed in a variety of organisms including protozoa (Shi et al., 2000), algae (Fuhrmann et al., 2001; Soupene et al., 2004), fungi (Liu et al., 2002), plants (Chuang and Meyerowitz, 2000; Smith et al., 2000; Waterhouse and Helliwell, 2003; Wesley et al., 2001), and metazoans (Brown et al., 2003; Dykxhoorn et al., 2003; Kennerdell and Carthew, 2000; Paddison and Hannon, 2002; Piccin et al., 2001; Tavernarakis et al., 2000). Common approaches rely on the production of dsRNA by transcription from transgenes that generate complementary (sense and antisense) transcripts or from a single transcription unit consisting of two inverted repeats (IRs) separated by a spacer (generating a hairpin-loop shaped RNA) (Dykxhoorn et al., 2003; Fuhrmann et al., 2001; Kennerdell and Carthew, 2000; Paddison and Hannon, 2002; Tavernarakis et al., 2000; Waterhouse and Helliwell, 2003). To avoid triggering antiviral/interferon responses, most constructs in mammals are designed to produce short hairpin RNAs, by expressing small IRs (19–29 nt) usually under the control of RNA polymerase III promoters (Brummelkamp et al., 2002; Paddison and Hannon, 2002; Paul et al., 2002). These approaches are useful for making permanent collections of hypomorphic epi-mutants that can be maintained in public repositories. Indeed, vectors designed for the simplified cloning of IR sequences by recombination are already being used for the high-throughput analysis of gene function in higher plants (Miki and Shimamoto, 2004; Waterhouse and Helliwell, 2003; Wesley et al., 2001).

One remaining problem, in a number of eukaryotes, is that the level of silencing triggered by dsRNA-producing transgenes is often quite variable, depending on the type of construct, transgene copy number, site of integration, and target gene (Brown et al., 2003; Chuang and Meyerowitz, 2000; Fuhrmann et al., 2001; Kalidas and Smith, 2002; Kerschen et al., 2004; Liu et al., 2002; Piccin et al., 2001; Waterhouse and Helliwell, 2003; Williams and Rubin, 2002). The use of IR constructs containing introns appears to improve their effectiveness (Kalidas and Smith, 2002; Smith et al., 2000), but independent transgenic lines still show variable phenotypes and degrees of target mRNA decrease (Brown et al., 2003; Waterhouse and Helliwell, 2003). Moreover, in *Arabidopsis thaliana*, reduction in transcript levels induced by IR transgenes appears to differ significantly among target genes (Kerschen et al., 2004). Thus, individual lines need to be molecularly characterized for suppression of a certain gene before potential phenotypic defects can be evaluated. This burdensome requirement is a drawback in high-throughput efforts, where it is desirable to inhibit reliably the expression of every target gene to screen for phenotypes of interest. To obviate this problem we undertook the development of constructs that would allow for direct selection of effective transgenic RNAi lines, using *Chlamydomonas* as a model system. We report here that tandem IR transgenes trigger simultaneous downregulation of both genes targeted by the construct. Thus, selection for an RNAi-induced phenotype permits the identification of transgenic lines showing effective interference of co-targeted genes.

Results

Inefficient RNA interference of a small multigene family

The small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), an enzyme essential for photosynthetic

CO_2 fixation, is encoded by two highly expressed genes (*RbcS1* and *RbcS2*) in *Chlamydomonas* (Goldschmidt-Clermont and Rahire, 1986; Khrebtukova and Spreitzer, 1996). Thus, robust RNAi would be required to observe a photosynthetic defect. In order to suppress the expression of this small multigene family we placed an IR, homologous to

the coding sequence of *RbcS1* and *RbcS2*, in the 3′UTR of the *ble* selectable marker (Lumbreras *et al.*, 1998), conferring resistance to bleomycin (zeocin) (Figure 1a). We reasoned that *ble-RbcS* IR transcripts would be spliced, exported to the cytosol, and translated for a limited period, permitting the selection of dsRNA-expressing transgenic strains. Although, Dicer-mediated processing of the 3′UTR dsRNA into siRNAs would eventually result in degradation of the remainder *ble* encoding ssRNA, which would lack a poly(A) tail. *Chlamydomonas* transformants were selected on zeocin-containing medium, supplemented with acetate as a carbon source, and then tested for their ability to grow and survive on minimal medium.

We recovered several zeocin-resistant transformants unable to grow on minimal medium (Figure 1b, strains 1, 7, and 13). This phenotype correlated with downregulation of expression of the Rubisco small subunit genes, both at the
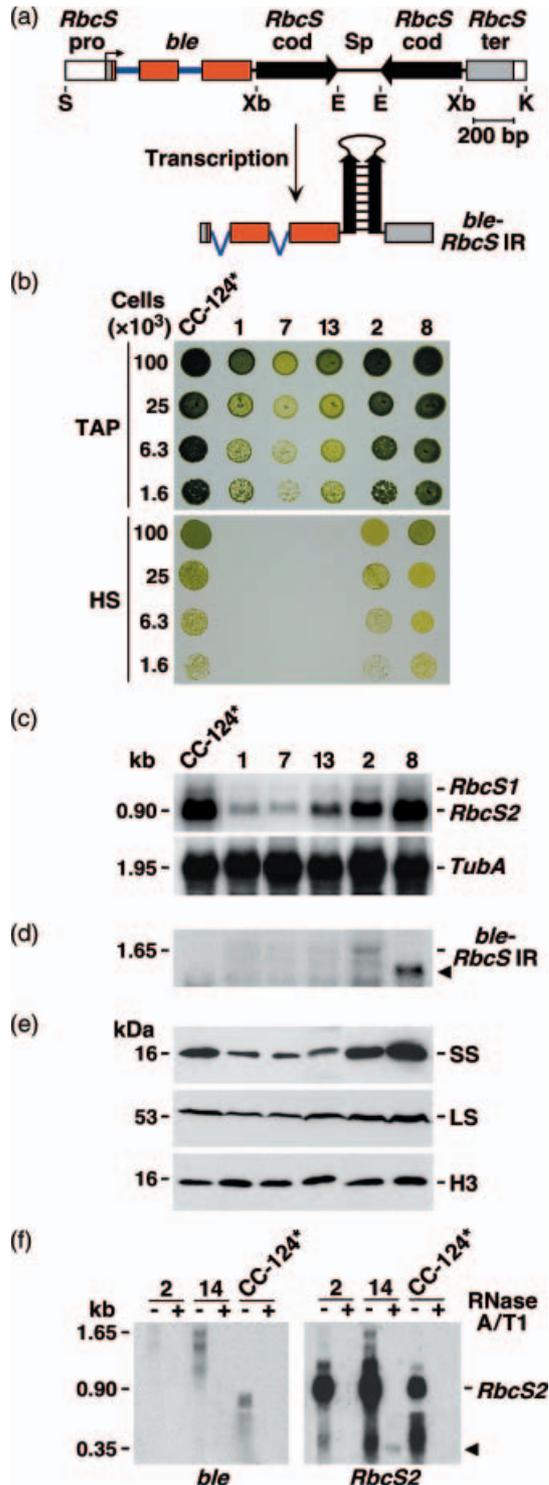
Figure 1. Effect of *RbcS* inverted repeat (IR) transgenes on the expression of the small subunit of Rubisco gene family.

(a) Schematic diagram of the construct (*ble-RbcS* IR) used to induce RNAi. A 350-bp fragment, corresponding to the *RbcS2* coding sequence (*RbcS* cod), was cloned in the forward and reverse orientations flanking a DNA spacer (Sp) in the 3′UTR (gray) of the *ble* selectable marker. The exons (red) and introns (blue) of the chimeric *ble* gene are indicated. The transgene is under control of the *RbcS2* promoter (*RbcS* pro) and terminator (*RbcS* ter) and is designed to generate, upon transcription, an RNA containing a double-stranded stem-loop structure in the 3′UTR. Restriction sites: E, *Eco*RI; K, *Kpn*I; S, *Sac*I; Xb, *Xba*I.

(b) Growth and survival of *ble-RbcS* IR transformants on Tris-acetate-phosphate (TAP) or high salt (HS) minimal media. Cells grown to logarithmic phase in TAP medium were diluted to the indicated numbers per 5 μl, spotted on plates, and incubated for 10 days under dim lights. CC-124*, strain transformed with the pSP124S plasmid (Lumbreras *et al.*, 1998) containing the *ble* selectable marker without an IR.
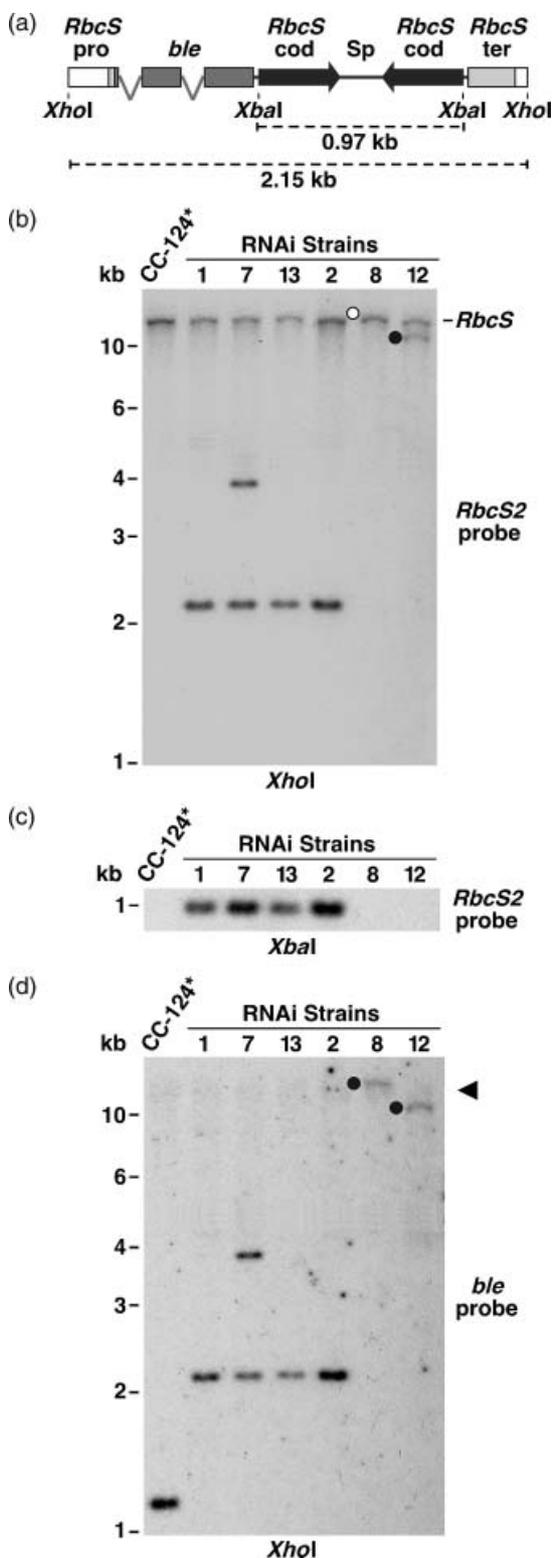
(c) Northern blot analysis of the strains described above. Total cell RNA was separated under denaturing conditions and hybridized to the *RbcS2* coding sequence (upper panel). Messages corresponding to each member of the Rubisco small subunit family are indicated. The same blot was re-probed with the coding sequence of the α-tubulin gene (*TubA*) as a control for equivalent sample loading (lower panel).

(d) Detection of *ble-RbcS* IR RNAs using as probe a ^{32}P-labeled PCR product corresponding to the *ble* coding sequence. Full-length, spliced transcripts are expected to be approximately 1.65 kb in size. The approximately 0.75 kb *ble* mRNA, in strain CC-124*, was cut off from the displayed panel. The faint signal at the bottom of some lanes corresponds to remaining radioactivity from the prior hybridization to *RbcS1*. The arrowhead indicates a truncated transcript in strain 8 (see text for details).

(e) Immunoblot analysis of total-cell proteins probed with a polyclonal antibody raised against the tobacco Rubisco holoenzyme. Both the nuclear encoded small subunit (SS, upper panel) and the chloroplast encoded large subunit (LS, middle panel) are detected. The blot was reprobed with an antibody against histone H3 as an indication of sample loading (H3, lower panel). Note that the CC-124* lane was underloaded when compared with the lanes of the RNAi strains.

(f) Detection of *RbcS2* dsRNA in transgenic strains. Total RNA isolated by a non-phenolic procedure was treated (+) or not (−) with a mix of RNase A/T1 to digest ssRNA. After denaturing gel electrophoresis and blotting, membranes were hybridized with the *ble* (left panel) or the *RbcS2* (right panel) coding sequences. Strains 2 and 14 show full-length and truncated *ble-RbcS* IR transcripts (left panel). Strain 14 also contains a ribonuclease-resistant fragment that hybridizes to *RbcS2* (but not to *ble*) and is of the expected size for the *RbcS2* dsRNA stem (right panel, arrowhead). The position of the endogenous, non-downregulated *RbcS2* mRNA is also indicated (right panel).

RNA (Figure 1c) and protein levels (Figure 1e). Transcripts corresponding to both members of the small multigene family were decreased (Figure 1c). Moreover, accumulation of the chloroplast-encoded large subunit of Rubisco was also somewhat reduced (Figure 1e, strains 1, 7, and 13), presumably because of translation and/or stability defects in the absence of the small subunit (Khrebtukova and Spreitzer, 1996). In effective RNAi strains *ble-RbcS* IR transcripts were virtually undetectable (Figure 1d, strains 1, 7, and 13), likely because of their rapid processing by Dicer and their subsequent degradation. However, photosynthetic deficiency showed low penetrance among the zeocin-resistant transformants. In several independent experiments, only approximately 1–3% of the transformants displayed a strict acetate requirement, whereas most strains showed minor growth defects (Figure 1b, strain 2) or behaved like the wild type (Figure 1b, strain 8).

Some phenotypically wild type transgenic strains arose from partial or complete deletion of the IR in integrated transgenes (Figure 2b–d, strains 8 and 12), resulting in the production of truncated *ble-RbcS* IR transcripts still containing a functional *ble* coding sequence (Figure 1d, strain 8 and data not shown). Other ineffective RNAi strains had integrated intact transgenes (Figure 2b–d, strain 2), but exhibited slight downregulation of the target transcript and protein (Figure 1c,e, strain 2). As postulated in other eukaryotes (Brown *et al.*, 2003; Kalidas and Smith, 2002; Piccin *et al.*, 2001), this may reflect position effects determining transcriptional silencing and low expression of transgenic dsRNA (below a threshold needed to trigger RNAi; Yang *et al.*, 2001). However, several strains showing minimal RNAi did produce discernible amounts of *ble-RbcS* IR RNA (Figure 1f left panel, strain 14 and data not shown). Moreover, treating purified RNA with a mix of RNase A/T1,

Figure 2. Analysis of integrated *ble-RbcS* inverted repeat (IR) transgenes in *Chlamydomonas* transformants exhibiting a range of photosynthetic phenotypes.

(a) Diagram of the construct used to induce RNAi. The sizes of expected DNA fragments resulting from digestion with *Xba*I or *Xho*I are indicated. Abbreviations as in Figure 1a.

(b) Southern blot analysis of genomic DNA from the indicated strains digested with *Xho*I and hybridized with the *RbcS2* coding sequence. The fragment corresponding to the endogenous duplicated *RbcS* genes is indicated. CC-124*, strain transformed with the pSP124S plasmid (Lumbreras *et al.*, 1998) containing the *ble* selectable marker without an IR.

(c) Southern blot analysis of genomic DNA digested with *Xba*I and hybridized with the *RbcS2* coding sequence. Strains 8 and 12 do not contain an intact *RbcS2* IR.

(d) Southern blot analysis of genomic DNA from the indicated strains digested with *Xho*I and hybridized with the *ble* coding sequence. The *ble* gene without a *RbcS2* IR corresponds to a *Xho*I fragment of approximately 1.18 kb (strain CC-124*). Although the integrated transgenes in strains 8 and 12 lack an intact *RbcS2* IR (Figure 2b,c), they still hybridize to the *ble* sequence (closed circles). The transgene in strain 12 also hybridizes to the *RbcS2* probe (Figure 2b, closed circle) suggesting that in this case the *RbcS2* IR was partially deleted at the 3′ end. By contrast, the transgene in strain 8 is not detected by the *RbcS2* probe (Figure 2b, open circle) suggesting that in this case the *RbcS2* IR was completely deleted (For comparison with Figure 2b, the arrowhead indicates the position of the endogenous *RbcS* fragment, which is smaller than the strain 8 *ble* hybridizing segment). However, as strain 8 is zeocin-resistant, a functional *ble* coding sequence (possibly transcriptionally fused to flanking *Chlamydomonas* sequences) must have been retained in the integrated transgene.

designed to degrade ssRNA while leaving dsRNA intact, revealed that some of these transformants contained dsRNA homologous to *RbcS2* (Figure 1f right panel, strain 14). Yet, this dsRNA failed to trigger RNAi possibly because its level is too low or because it accumulates in a compartment and/or in a certain conformation not accessible for processing by Dicer.

Direct selection of transgenic strains showing an RNAi-induced phenotype

Because of the wide range of problems associated with inefficient transgenic RNAi strains, we designed a construct to select directly for an RNAi-induced phenotype. In many tryptophan-producing organisms, tryptophan synthase β-subunit (TSB) converts the indole analog 5-fluoroindole (5-FI) into the toxic tryptophan analog 5-fluorotryptophan. Mutants defective in TSB are therefore resistant to 5-FI (Palombella and Dutcher, 1998). To silence *Maa7* (encoding *Chlamydomonas* TSB; Palombella and Dutcher, 1998), we placed an IR homologous to its 3′UTR in the 3′ end of the *ble* marker. *Chlamydomonas* transformants were selected on medium containing both zeocin and 5-FI. Despite relatively low transformation efficiency, all recovered transformants exhibited resistance to 5-FI (Figure 3a) and showed reduced levels of the *Maa7* transcripts (Figure 3b). Consistent with this decrease resulting from RNAi-induced mRNA degradation, we detected siRNAs hybridizing to the IR *Maa7* sequence in strains carrying an integrated transgene but not in the wild type (Figure 3c). These small RNAs corresponded to both sense and antisense polarities (Figure 3d), as previously reported for several plant species (Hamilton and Baulcombe, 1999). However, there does not appear to be a strict correlation between the steady-state level of siRNAs and the degree of *Maa7* gene silencing (Figure 3a,c).

Co-silencing of target genes by RNAi with tandem inverted repeat transgenes

In metazoans, several unrelated genes can be silenced simultaneously by co-injection of the cognate dsRNAs (Dudley *et al.*, 2002; Schmid *et al.*, 2002). Therefore, we examined whether a tandem IR construct (Figure 4a) would downregulate expression of both *Maa7* and another gene of interest. We tested for co-silencing of *Maa7* and the genes encoding either the *Chlamydomonas* homolog of the Ku80 DNA repair protein (*Ku80*), the RNA-binding protein 1 (*Rbp1*), or cytosolic thioredoxin h1 (*Trxh1*). As in the case of *Ku80*, we wished to study the sensitivity of RNAi strains to DNA damaging agents (such as zeocin), the new construct was modified to remove most of the *ble* coding region (Figure 4a). We also placed downstream from the tandem IR transcription unit the aminoglycoside 3′-phosphotransferase gene (Sizova *et al.*, 2001), conferring resistance to
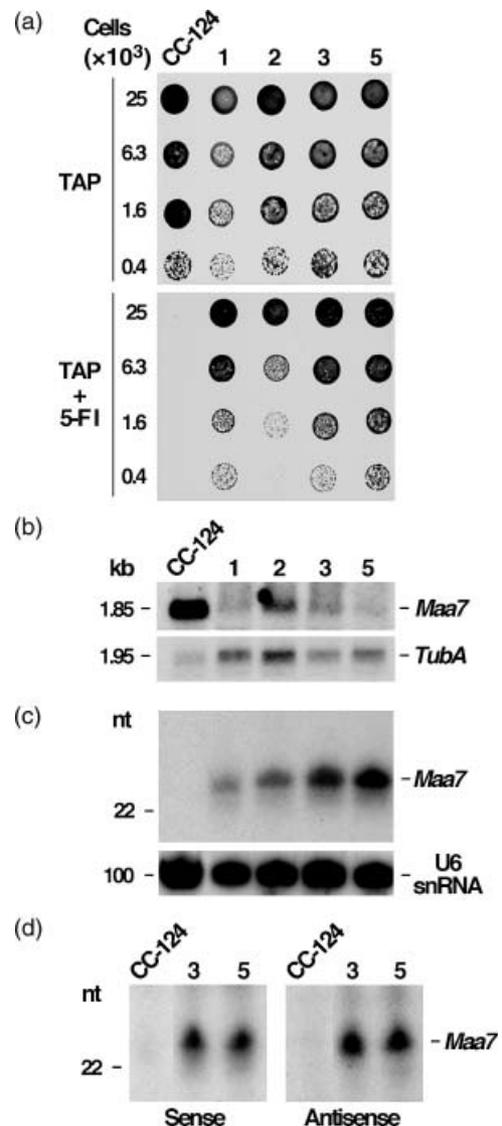
Figure 3. Silencing of tryptophan synthase β subunit by expression of *Maa7* inverted repeat (IR) transgenes in transformants selected on 5-fluoroindole-containing medium.
Transgenic strains were generated by transformation with a construct similar to *ble-RbcS* IR (Figure 1a) but replacing the *RbcS2* IRs with 450 bp IR fragments corresponding to the 3′UTR of *Maa7*.
(a) Growth and survival of *ble-Maa7* IR transformants on TAP medium without (upper panel) or with (lower panel) 10 μM of 5-fluoroindole and supplemented with 1.5 mM L-tryptophan. CC-124, untransformed wild type strain.
(b) Northern blot analysis of total cell RNA sequentially hybridized with ^{32}P-labeled PCR products corresponding to the coding sequence of *Maa7* (upper panel), to evaluate the degree of mRNA reduction, or the coding sequence of *TubA* (lower panel) to assess the amount of sample loaded per lane.
(c) Detection of siRNAs in transgenic strains undergoing *Maa7* silencing. Column-fractionated small RNAs were separated in a 15% denaturing polyacrylamide gel, electroblotted onto a nylon membrane, and hybridized with the *Maa7* 3′UTR sequence (upper panel). The same filter was re-probed with the U6 small nuclear RNA (U6 snRNA) sequence as a control for equal loading of the lanes (lower panel).
(d) To examine the polarity of the siRNAs, identical blots were hybridized with *in vitro* transcribed *Maa7* 3′UTR riboprobes detecting either sense (left panel) or antisense (right panel) RNAs.
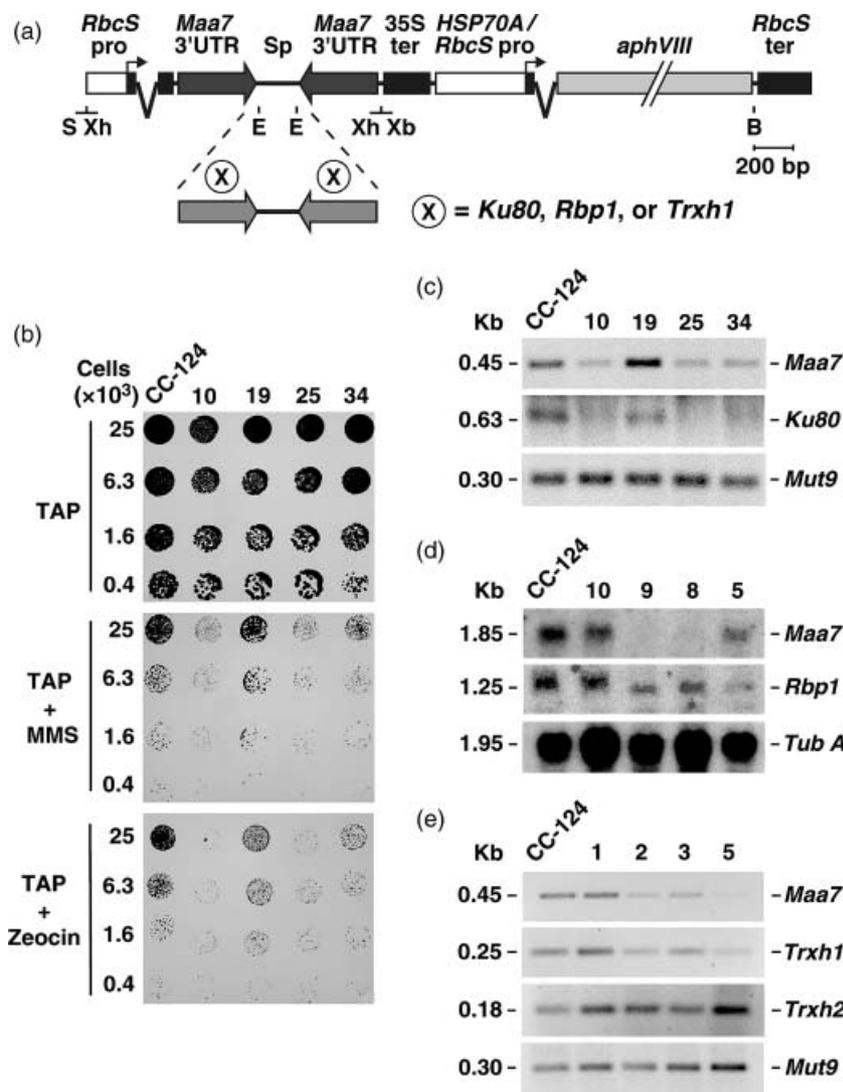
Figure 4. Co-silencing of tryptophan synthase β subunit and another target gene (X) induced by the expression of tandem *Maa7/X* inverted repeat (IR) transgenes. Gene X encodes either the DNA repair protein Ku80 (*Ku80*), the RNA-binding protein 1 (*Rbp1*), or cytosolic thioredoxin h1 (*Trxh1*).

(a) Diagram of the construct used to trigger RNAi and generated by modification of the *ble-Maa7* IR transgene. The engineered *ble* sequence was deleted, except for the first intron and a few surrounding nucleotides. Fragments corresponding to target gene X were cloned in sense and antisense orientation, flanking a DNA spacer (Sp), in between the arms of the *Maa7* 3'UTR IR. The *Maa7/X* IR transgene is under control of the *RbcS2* promoter (*RbcS* pro) and the *Cauliflower Mosaic Virus 35S* terminator (35S ter) and is designed to generate, upon transcription, an RNA folding into a hairpin-loop structure with a stem of tandem IRs. The previously engineered aminoglycoside 3'-phosphotransferase (*aphVIII*) gene (Sizova *et al.*, 2001) was placed immediately downstream from the *Maa7/X* IR transcription unit. Restriction sites: B, *Bam*HI; E, *Eco*RI; S, *Sac*I; Xb, *Xba*I; Xh, *Xho*I.

(b) Growth and survival of *Maa7/Ku80* IR transformants on plates containing TAP alone or TAP plus the DNA damaging agents methylmethane sulfonate (MMS, 2 mм) or zeocin (1.5 µg ml^{-1}). CC-124, untransformed wild type strain.

(c) Semi-quantitative reverse transcriptase (RT)-PCR analysis of the *Maa7/Ku80* IR transgenic strains. Reactions were performed as described under Experimental procedures. The upper panel shows a reverse image of agarose separated RT-PCR products amplified from *Maa7* transcripts. The middle panel shows RT-PCR products amplified from *Ku80* mRNAs and detected by Southern hybridization with a *Ku80* specific probe. Amplification of the mRNA corresponding to *Mut9* (encoding a serine/threonine protein kinase) was used as a control for equal amounts of input RNA and for the efficiency of the RT-PCRs (lower panel).

(d) Northern blot analysis of the *Maa7/Rbp1* IR transformed strains. Electrophoretically fractionated and blotted total cell RNA was sequentially hybridized with ^{32}P-labeled PCR products corresponding to the coding sequences of *Maa7* (upper panel), *Rbp1* (middle panel), or *TubA* (lower panel).

(e) Semi-quantitative RT-PCR assays on total RNA samples from *Maa7/Trxh1* IR transformants. The panels display reverse images of agarose-fractionated RT-PCR products corresponding to the indicated genes. *Trxh2*, gene encoding cytosolic thioredoxin h2.

paromomycin. This allowed the selection of transformants on medium containing both 5-Fl and paromomycin, to avoid the isolation of spontaneous mutants defective in TSB.

Our initial screens used a relatively low selection pressure (i.e. a sublethal 5-Fl concentration) to obtain RNAi strains with different degrees of silencing of *Maa7*.

This way we were able to examine the correlation of *Maa7* suppression with interference of other genes targeted by the same tandem construct. Transformants containing integrated copies of the *Maa7/Ku80* IR transgene were sensitive to radiomimetic DNA damaging agents such as methylmethane sulfonate and zeocin (Figure 4b). This sensitivity correlated with the level of reduction of the *Ku80* transcripts (Figure 4c), as expected for a role of Ku80 in the repair of DNA double-strand breaks (Tamura *et al.*, 2002). In addition, the *Ku80* RNA reduction was generally proportional to the decrease in the *Maa7* transcripts (Figure 4c), suggesting that both messages were targeted simultaneously by RNAi induced by the tandem IR transgene. Notably, we also observed correlation between the strength of the phenotypes. When transformants were tested for survival at higher concentrations of 5-Fl (from 7 to 20 μM), RNAi strains with the highest resistance to 5-Fl displayed the greatest sensitivity to radiomimetic agents (data not shown).

The other two genes tested, *Rbp1* and *Trxh1*, also displayed co-silencing with *Maa7* when targeted by a tandem IR transgene (Figure 4d,e). Based on the analysis of 40 independent RNAi strains (Figure 4 and data not shown), only one transformant (Figure 4d, strain 5) showed lack of correlation between downregulation of expression of *Maa7* and that of another gene targeted by the same tandem IR construct. In this case, the integrated transgene underwent a rearrangement with partial deletion of the second arm of the *Maa7* IR (data not shown). This most likely results in the production of a shorter *Maa7* dsRNA and a lower degree of TSB silencing relative to comparable strains carrying intact transgenes. However, because the IR corresponding to the gene of interest is internal to the arms of the *Maa7* repeat (Figure 4a, gene X), the targeted *Rbp1* gene was still efficiently suppressed (Figure 4d).

These experiments demonstrated that tandem IR transgenes induce co-silencing of both targeted genes. However, as the degree of specificity of RNAi induced by tandem IR transgenes is unknown, we also examined the expression of unrelated endogenous genes in *Chlamydomonas* RNAi strains. We detected no effect on the accumulation of transcripts encoding α-tubulin (Figure 4d), the 18S ribosomal RNA, and the U6 snRNA (data not shown). Moreover, cytosolic thioredoxin h2 (*Trxh2*) (Lemaire *et al.*, 2003) mRNA levels remained unperturbed in strains undergoing RNAi of the closely related *Trxh1* gene (Figure 4e). Thus, our results suggest that gene silencing induced by this method results in specific loss of target gene expression without perturbing overall gene expression.

Discussion

Robust transgenic RNAi technology should contribute to the functional analysis of accumulating genome sequences.

RNAi is highly sequence specific and a loss-of-function phenotype can be readily linked to a given gene (Kusaba, 2004; Mittal, 2004; Waterhouse and Helliwell, 2003). In addition, RNAi can be used for the simultaneous suppression of multigene family members as well as redundant genes in polyploid species (Lawrence and Pikaard, 2003). As RNAi only knocks down target gene expression, it also offers an advantage in performing functional genomic studies on essential genes (Hemann *et al.*, 2003). Moreover, inducible RNAi systems are being developed in both higher plants (Chen *et al.*, 2003; Guo *et al.*, 2003) and animals (Mittal, 2004). Lastly, phenotypic suppression by integrated IR transgenes is inherited stably in a number of eukaryotes (Carmell *et al.*, 2003; Kusaba, 2004; Soupene *et al.*, 2004), validating this technology for the generation of collections of stable epi-mutants.

However, there are also concerns and limitations in the use of RNAi. In *C. elegans*, the degree of knockdown achieved by transient RNAi shows considerable experimental variation (Mittal, 2004; Simmer *et al.*, 2003). Moreover, in both worms and mammals, insufficient RNAi silencing of a gene might not generate an observable phenotype, thereby contributing to a significant false-negative rate in genome-wide screens (Paddison *et al.*, 2004; Simmer *et al.*, 2003). In higher plants and algae, the extent of suppression triggered by IR transgenes is variable and some target genes show little or no silencing (Chuang and Meyerowitz, 2000; Fuhrmann *et al.*, 2001; Kerschen *et al.*, 2004; Waterhouse and Helliwell, 2003). A recent study in *Arabidopsis* indicated that independent, single copy RNAi lines targeting the same gene reduced target transcript levels to a similar degree (Kerschen *et al.*, 2004). In contrast, multiple copy RNAi lines showed much more variability, perhaps associated with differences in the transcriptional silencing of the IR transgenes. This susceptibility to transcriptional silencing may also compromise the usefulness of these lines over multiple generations (Kerschen *et al.*, 2004). However, even in the case of single copy RNAi strains, some genes were refractory to silencing. Interestingly, in *C. elegans* (Cutter *et al.*, 2003) and to some extent in *Arabidopsis* and tomato (Han *et al.*, 2004; Kerschen *et al.*, 2004), strong RNAi effects appear to correlate with high expression levels of the targeted genes.

We have explored the effectiveness of IR transgenes to downregulate the expression of a small multigene family, encoding the small subunit of Rubisco, in the green alga *C. reinhardtii*. Our results indicated a number of problems associated with variable transgenic RNAi effects. Some strains showed defective transgene integration resulting in partial or complete deletion of the IR sequences. Other strains displayed low levels of dsRNA expression, presumably due to transcriptional silencing of the integrated IR transgenes. As reported in *Arabidopsis* (Kerschen *et al.*, 2004), this occurred frequently in multiple copy transformants (data not shown) but was also observed in

single copy RNAi lines. Intriguingly, some strains produced detectable levels of dsRNA but failed to induce an RNAi phenotype. We speculate that in these cases the dsRNA may accumulate in a cellular compartment or in a particular conformation (for instance in a nucleoprotein particle) that prevents its processing into siRNAs by Dicer (Cerutti, 2003). For the study of individual genes, these problems can be overcome by screening multiple independent transgenic lines for suppression of target gene expression. However, for high-throughput studies, uncertainty about the suppressive effect of IR constructs represents a serious hindrance. This prompted us to develop a system for the direct selection of effective transgenic RNAi strains.

Silencing of *Maa7*, encoding tryptophan synthase β subunit, allows *Chlamydomonas* cells to grow on medium containing 5-Fl (Palombella and Dutcher, 1998). Tandem IR constructs were designed to suppress simultaneously *Maa7* and another target gene of interest, allowing direct selection of effective RNAi lines in the presence of 5-Fl. For three tested genes, encoding the *Chlamydomonas* Ku80 homolog, an RNA-binding protein, and cytosolic thioredoxin h1, downregulation of *Maa7* correlated strictly with suppression of the co-targeted gene and the appearance of the expected phenotypes. Co-silencing of unrelated genes has also been achieved in *C. elegans*, *Drosophila melanogaster*, and mammalian cells by transient RNAi with pools of dsRNA or hairpin siRNA expression vectors (Dudley *et al.*, 2002; Longman *et al.*, 2001; Schmid *et al.*, 2002; Yu *et al.*, 2003). In addition, the genes encoding proliferating cell nuclear antigen and a subunit of magnesium chelatase have been simultaneously suppressed in *Nicotiana benthamiana* using a geminivirus-derived vector (Peele *et al.*, 2001). Thus, the use of tandem IR transgenes to silence unrelated genes is likely to be broadly applicable. However, the limitations of the technique for targeting more than two genes remain to be explored as the RNAi machinery is saturable with excess dsRNA in both *C. elegans* and *Drosophila* (Parrish *et al.*, 2000; Yang *et al.*, 2001).

The genes suppressed by tandem IR RNAi in *Chlamydomonas* correspond to a wide range of expression levels. *Ku80* is normally expressed at very low levels, *Rbp1* at low to moderate levels, and *Trxh1* at moderate levels. Moreover, we have now silenced over 10 genes using tandem IR transgenes; including one encoding a SET domain containing protein that had been recalcitrant to suppression with single IR transgenes (K. van Dijk and Heriberto Cerutti, unpublished data). In addition, the technique is flexible. The use of low (sublethal) selective pressure allows the isolation of transgenic strains with different degrees of silencing (an epi-allelic series for phenotypic characterization). This strategy will likely be useful for recovering transgenic RNAi strains targeting essential genes. Conversely, the use of high (lethal) selective pressure allows the identification of transgenic strains showing only strong silencing of the

target genes. In summary, selectable tandem IR transgenes should provide a way to exploit the rapidly accumulating genome sequence of *C. reinhardtii*. Because only a short segment is needed for interference, partial open reading frame identification will suffice to test sequences of interest for desirable phenotypes. However, additional negative selectable markers will need to be developed as *Maa7* will not be suitable for the analysis of genes involved in amino acid metabolism. Modification of the tandem IR system described here, to silence readily observable reporters or other genes with a selectable RNAi phenotype, may also simplify the generation of effective, stable RNAi lines in higher eukaryotes.

Experimental procedures

Plasmid construction

For generating the *ble-RbcS* IR transgene (NE-445) (Figure 1a), a 350-bp fragment corresponding to the coding sequence of *RbcS2* (differing only in eight nucleotides from the *RbcS1* coding sequence; Goldschmidt-Clermont and Rahire, 1986) was amplified by PCR from a cloned cDNA template with primers Rbcs2-cod-5(P/X) [5'-gccctgcagtctagaGTCATTGCCAAGTCCTCCGTCT-3', adding *Pst*I and *Xba*I sites (underlined)] and Rbcs2-cod-3(B/H) [5'-cgcggatccaagcttATGGTCCAGTAGCGGTTGTCGTAG-3', adding *Bam*HI and *Hin*dIII sites (underlined)]. The PCR product was cloned in forward and reverse orientations into the *Pst*I/*Bam*HI and *Hin*dIII/*Xba*I sites of the pSTBlue-1 vector (Novagen, Madison, WI, USA), flanking a 200-bp DNA spacer previously inserted into the *Eco*RV site. Plasmid pSP124S, carrying the chimeric *ble* selectable marker (Lumbreras *et al.*, 1998), was obtained through the *Chlamydomonas* Genetics Center (Duke University) and modified to destroy the *Xba*I and *Eco*RI sites preceding the *RbcS2* promoter. The *RbcS2* IR cassette was then excised from pSTBlue-1 by *Xba*I digestion and inserted into the corresponding site of pSP124S, in the *ble* 3'UTR (Figure 1a). A similar strategy was used to construct the *ble-Maa7* IR transgene (NE-498). A 450-bp fragment corresponding to the *Maa7* 3'UTR (Palombella and Dutcher, 1998) was amplified by PCR from genomic DNA with primers Trp-SB-5 (5'-GCACTGTGCTTTGACAGACAAG-3') and Trp-SB-3 (5'-CGATTGGTAGCAACAAAGTGAG-3'). The PCR product was cloned in forward and reverse orientations, by blunt end ligation, into the *Bam*HI and *Hinc*II sites of the pSTBlue-1 vector (Novagen), flanking the 200 bp DNA spacer. This *Maa7* IR cassette was then excised by digestion with *Sph*I/*Hin*dIII and cloned, by blunt end ligation, into the *Xba*I site of the modified pSP124S plasmid.

For generating the *Maa7/X* IR transgene (Figure 4a), the previously engineered *aphVIII* gene was excised from plasmid pSI103 (a gift from M. Fuhrmann; Sizova *et al.*, 2001) by digestion with *Sac*I/*Kpn*I and cloned, by blunt end ligation, into the *Kpn*I site of modified pSP124S. This placed the *aphVIII* transcription unit immediately downstream from the *ble* marker. We then deleted most of the chimeric *ble* sequence (except for the first intron) by complete digestion with *Aat*II (site located inside the *ble* coding sequence) and partial digestion with *Xho*I (site located immediately downstream from the *RbcS2* terminator). The *Maa7* IR cassette (see above) was cloned into this vector, by blunt end ligation, replacing the deleted sequence. Lastly, a 180-bp fragment containing the *Cauliflower Mosaic Virus 35S* terminator from plasmid pPTN134 (a gift from T. Clemente) was inserted downstream from the *Maa7* repeats to

create the *Maa7/X*IR transgene (NE-537). This vector contains unique *Eco*RI sites flanking the spacer in between the *Maa7* IR arms for the cloning of IR cassettes targeting any gene of interest (Figure 4a). The sequence of the *Maa7/X* IR transgene is available as Genbank accession number AY710294 and the plasmid is available from the Chlamydomonas Genetics Center at Duke University.

The *Trxh1* IR cassette was constructed by PCR amplification from a full-length cDNA clone (a gift from S. Lemaire; Stein *et al.*, 1995) of a 450-bp fragment corresponding to the *Trxh1* 3′UTR with primers Trx-5 (5′-GGAAAGGGTTGGTGTTGTTG-3′) and Trx-3 (5′-AA-TTACAAGGGCCGATTGC-3′). The PCR product was cloned in sense and antisense orientations, by blunt end ligation, into the *Bam*HI and *Hin*dIII sites of the pSTBlue-1 vector (Novagen) flanking the 200 bp DNA spacer. This *Trxh1* IR cassette was then excised by digestion with *Pst*I/*Xba*I and inserted, by blunt end ligation, into the *Eco*RI sites of *Maa7/X* IR to generate the *Maa7/Trxh1* IR transgene. *Ku80* and *Rbp1* IR cassettes were constructed by similar recombinant DNA manipulations of approximately 1.1 kb fragments excised from cDNA clones. The IRs were then cloned into the *Eco*RI sites of *Maa7/X* IR to produce the *Maa7/Ku80* IR and *Maa7/Rbp1* IR transgenes. The *Ku80* sequence is available as accession number AV393990 and the *Rbp1* sequence is available from the *Chlamydomonas* genome project (C_240124; http://genome.jgi-psf.org/chlre2/chlre2.home.html). Although the RNAi machinery is saturable with excess dsRNA in both *C. elegans* and *Drosophila* (Parrish *et al.*, 2000; Yang *et al.*, 2001), we have achieved successful co-silencing with IR arms corresponding to gene X (Figure 4a) between 0.5 and 2.5-fold the length of each *Maa7* repeat (450 bp).

Culture conditions, transformation, and selection procedures

Unless noted otherwise, *C. reinhardtii* cells were grown in Tris-acetate-phosphate (TAP) medium (Harris, 1989). Plasmid DNA linearized with *Sac*I was used in all transformation experiments. The wild type strain CC-124 (mt⁻) (Harris, 1989) was transformed by the glass beads procedure (Kindle, 1990) and allowed to recover for 2 days, to permit induction of RNAi, before plating under selective conditions. *ble-RbcS* IR tranformants were selected on TAP medium containing 5 μg ml⁻¹ zeocin (Invitrogen, Carlsbad, CA, USA). *ble-Maa7* IR transgenic strains were isolated on TAP medium supplemented with 1.5 mM L-tryptophan, 2 μg ml⁻¹ zeocin, and 7 μM 5-Fl (Sigma). *Maa7/X*IR transformants were selected on TAP medium containing 1.5 mM L-tryptophan, 5 μg ml⁻¹ paromomycin, and 5 μM 5-Fl. Plates were incubated under dim lights (approximately 50 μmol m⁻² sec⁻¹ photosynthetically active radiation) covered with one layer of paper towels (Palombella and Dutcher, 1998). Isolated transgenic strains were kept under constant selective pressure to circumvent any silencing of integrated IR transgenes. Transformants recovered in 5-Fl-containing medium, via downregulation of *Maa7*, were also tested for resistance to 5-methylanthranilate and sensitivity to the tryptophan analog 5-methyltryptophan, as expected for epi-mutants defective in TSB (Palombella and Dutcher, 1998). For phenotypic analyses, cells grown to logarithmic phase in TAP medium were serially diluted, spotted on plates of the appropriate media (see figure legends), and incubated for 10–15 days under dim lights (Zhang *et al.*, 2002).

DNA and RNA analyses

Standard protocols were used for genomic DNA isolation, fractionation by gel electrophoresis, and Southern hybridization (Sambrook and Russell, 2001; Wu-Scharf *et al.*, 2000). Total cell RNA was purified with TRI Reagent (Molecular Research Center,

Cincinnati, OH, USA), according to the manufacturer's instructions. The isolated RNA was separated by agarose/formaldehyde gel electrophoresis, blotted onto nylon membranes, and hybridized with ³²P-labeled probes (Sambrook and Russell, 2001; Wu-Scharf *et al.*, 2000; Zhang *et al.*, 2002). For the detection of dsRNA, samples were purified by a non-phenolic procedure (Plant RNeasy; Qiagen, Valencia, CA, USA), treated with RNase A/T1 as described in the high-speed hybridization RNase protection assay (Ambion, Austin, TX, USA), and subjected to Northern blot analysis. In order to assay for the presence of small RNAs, TRI Reagent isolated total RNA was fractionated through Microcon YM-100 centrifugal devices (Millipore, Billerica, MA, USA) to remove high-molecular weight transcripts. Small RNAs were concentrated from the filtrate by ethanol precipitation, resolved in 15% polyacrylamide/7 M urea gels, and electroblotted to Hybond-XL membranes (Hamilton and Baulcombe, 1999). Blots were hybridized with ³²P-labeled DNA probes at 35°C for 48 h using the High Efficiency Hybridization System (Molecular Research Center). Strand-specific (sense or antisense) riboprobes were generated by *in vitro* transcription with the Maxiscript kit (Ambion).

Protein analysis

Approximately 5×10^6 cells, grown to logarithmic phase, were pelleted by centrifugation and resuspended in 50 μl of SDS-gel running buffer (Sambrook and Russell, 2001). Five-microliter aliquots of boiled samples were separated by 15% SDS-PAGE and electrophoretically transferred to nitrocellulose membranes (Sambrook and Russell, 2001). The Rubisco subunits were immunodetected by overnight incubation at 4°C with a 1:100 000 dilution of a rabbit antibody raised against the tobacco holoenzyme (a generous gift from R. Chollet). To evaluate the loading of the lanes we used a polyclonal anti-histone H3 antibody (Abcam, Cambridge, MA, USA ab1791) at a 1:5000 dilution. After incubation with a goat anti-rabbit secondary antibody, conjugated to horseradish peroxidase, a chemiluminescent substrate (Pierce, Rockford, IL, USA) was used for autoradiographic detection.

Reverse transcriptase (RT)-PCR analysis

TRI Reagent isolated total RNA was treated with DNase I (DNA-free; Ambion) to remove contaminating DNA and reverse transcription reactions were carried out as previously described (Carninci *et al.*, 1998). Synthesized cDNA was then used as a template in standard PCR reactions (Sambrook and Russell, 2001; Wu-Scharf *et al.*, 2000). The numbers of cycles showing a linear relationship between input RNA and the final product were determined in preliminary experiments. All primers were designed to match exonic sequences flanking one or more introns to distinguish contaminating PCR products generated by amplification of possibly remaining DNA. Controls also included the use as template of reactions without RT and verification of PCR products by hybridization with specific probes (data not shown). The PCR conditions for amplification of *Trxh1*, *Maa7*, and *Mut9* were 25 cycles at 93°C for 30 sec, at 55°C for 30 sec, and at 71°C for 60 sec. The *Trxh2* PCR product was amplified by 34 cycles under the same conditions, except for annealing at 53°C. PCR reactions for detection of *Ku80* were carried out for 38 cycles at 93°C for 30 sec, at 58°C for 30 sec, and at 71°C for 75 sec. Five-microliter aliquots of each RT-PCR reaction were resolved on 1.5% agarose gels and visualized by ethidium bromide staining, except for the *Ku80* PCR products which were detected by Southern hybridization. The primer sequences were as follows: Trxh1-F, 5′- ATGCTGGCAAGGTCATCTTC-3′; Trxh1-R, 5′-CAACAACACCAA-CCCTTTCC-3′; Maa7-F, 5′-TGAACATCACTGCCCCTACTC-3′; Maa7-R,

5′-CCCAGCGAGTTGTTGATCTTA-3′; Mut9-F(5), 5′-GCTGTACATCT-CGTGCGTGT-3′; Mut9-R(2), 5′-ATGGCGGTCACGTAGAAGC-3′; Trxh2-F, 5′-TGTGCGCTAACTCGAACAAC-3′; Trxh2-R, 5′-CCTCAA-TAGCAGCGAGCAT-3′; Ku80-F, 5′-ACGGCGGAGGAAGACTTT-3′; and Ku80-R, 5′-TGCATCGTGATAGCACCAATA-3′.

Acknowledgements

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