Theor Appl Genet (1999) 98: 371–378

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Inheritance, expression, and silencing of a chitinase transgene in rice*

Received: 24 April 1998 / Accepted: 18 August 1998

Abstract The inheritance and expression of a transgene locus consisting of multiple copies of a rice chitinase gene under the control of the CaMV 35S promoter was studied in the T_3 and T_4 generations of a transformed line that expressed the chitinase at a high level. All T_3 progeny of a homozygous T_2 parent expressed the chitinase constitutively at 3 weeks after germination, but a proportion of the progeny had undetectable levels of chitinase 8 weeks after germination, indicating silencing of the transgene. Transgene silencing was also observed among progeny of a hemizygous parent. However, we did not observe chitinase gene silencing among progeny of another homozygous line that expressed the transgenic chitinase at a five- to tenfold lower level. Thus, expression level, rather than copy number, of the transgene appears to be critical for silencing. Silencing was observed in the leaf, sheath, and root tissues of the plant, indicating that it is not restricted to specific tissues. Silencing was first observed in the youngest leaves and only later in the oldest leaves of the same plant. There was co-silencing of the selectable marker gene, *hpt*, which is also driven by the CaMV 35S promoter. Unlike the two transgenes (chitinase and marker), the resident homologous chitinase gene with seed-specific expression and two nonhomologous chitinase genes induced in the leaves upon pathogen infection were not silenced. The silent phenotype was inherited in the T_4 generation plants, while progeny of expressing plants exhibited silencing. The chitinase transgene appeared intact, and no evidence for gross alterations or methylation of CCGG sites was found. The silent phenotype could not be reversed by treatment with 5-azacytidine. Northern blot analysis and nuclear run-on transcription studies indicated that silencing occurred at the transcriptional level. The implications of transgene silencing in genetic engineering of monocot plants for disease resistance are discussed.

Key words Chitinase · Gene-silencing · *hpt*-gene-silencing · Rice · Transcriptional silencing

*Contribution no. 98-284-J from the Kansas State Agriculture Experiment Station

Communicated by M. A. Saghai Maroof

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Introduction

Inheritance and stable expression of transgenes is an important concern in crop improvement through gene manipulations. With the successful development of procedures for transformation, transgenic plants and their progeny have been studied for continued expression of the foreign gene through several generations. These studies have revealed that in some cases the transgene expression was lost in a variable proportion of the progeny. This phenomenon, referred to as "gene silencing", has been studied most extensively in dicot plants, such as tobacco, petunia, tomato, and buckweed (reviewed in: Matzke and Matzke 1995; Meyer 1995; Stam et al. 1997). In general, transgene suppression and the associated cosuppression of

homologous host genes have been observed in plants with multiple copies and/or high-level expression of the transgene. Two types of gene silencing have been recognized, namely transcriptional and post-transcriptional silencing, both of which lead to reduced or undetectable steady state levels of transcripts. In some cases, gene silencing has been shown to be associated with methylation of promoter and/or coding regions of the target genes (Matzke et al. 1989; Ingelbrecht et al. 1994). Several mechanisms/models have been proposed to explain the phenomena of gene silencing and cosuppression (Finnegan and McElroy 1994; Matzke and Matzke 1995; Meins and Kunz 1994; Meyer 1995; Stam et al. 1997). These include methylation of specific sequences in the promoter region, antisense or aberrant RNA production, ectopic gene (or RNA/DNA) pairing leading to methylation or heterochromatinization of the target gene, and a RNA threshold model. It is possible that there are multiple mechanisms for gene silencing, which is often stochastic.

In contrast to the extensive studies on gene silencing in dicotyledonous plants, there have been relatively few studies on gene silencing in monocotyledonous plants or on the mechanisms of transgene inactivation (Cooley et al. 1995; Rathore et al. 1993; Register et al. 1994). A recent study of three independent rice transgenic lines demonstrated silencing of the *bar* gene in one line in the R_1 and R_2 generations and provided evidence for the involvement of methylation in transgene silencing (Kumpatla et al. 1997). We have successfully introduced a rice chitinase gene under the control of the cauliflower mosaic virus (CaMV) 35S promoter into rice and obtained high-level expression of this gene in T_0 and T_1 generation plants (Lin et al. 1995). The high-level, constitutive expression of chitinase resulted in greater resistance to sheath blight, a disease caused by the fungus, *Rhizoctonia solani*. In the investigation presented in this paper, we studied the expression of this chitinase transgene up to the T_4 generation. Silencing of the chitinase transgene and of the selectable marker *hpt* (hygromycin phosphotransferase) gene was observed in about 23% of the progeny in the T_3 and T_4 generations. While several features of gene silencing in our transgenic rice plants were similar to those reported for silencing of the chitinase gene in tobacco (Kunz et al. 1996) and of th *bar* gene in rice (Kumpatla et al. 1997), there were significant differences in the mechanism of silencing and in the transmission of the silent phenotype to the next generation.

Materials and methods

Plant materials

The two transgenic rice plants nos. 354 and 178 expressing the rice chitinase gene constitutively were obtained by polyethyleneglycol-mediated DNA uptake by rice protoplasts, characterized and selfed to obtain seeds (Lin et al. 1995).

Preparation of protein extracts from transgenic rice plants

Fresh tissue (0.2 g) collected from leaves, sheaths, or roots of transgenic or untransformed control plants grown in the greenhouse were frozen in liquid nitrogen in a small mortar and ground to a fine powder. The powdered material was transferred to Eppendorf tubes with 0.5 ml of extraction buffer (0.05 *M* TRIS-HCl, pH 7.0, and 10% glycerol) containing 1 m*M* PMSF (phenylmethylsulfonyl fluoride). The mixture was centrifuged at 10,000 rpm for 5 min, and the supernatant was collected. The protein concentrations of the extracts were determined by using a modified bicinchoninic acid reagent (Pierce, Rockford, Ill.) with bovine serum albumin as standard, following the supplier's protocol.

Analysis of chitinase transgene expression

The transgenic plants derived from the homozygous parent 354-7-1 (T_2 plant) and hemizygous parent 354–5-5 (T_2 plant) were analyzed for expression of the transgenic chitinase by Western blot using anti-bean chitinase antibody as described by Lin et al. (1995) at 3-week intervals starting from 3 weeks after germination. Leaf tissues were sampled unless otherwise stated.

Southern blotting

Genomic DNAs were isolated as described by Murray and Thompson (1980) from rice leaf tissue, and 10-μg aliquots were digested with *Hin*dIII and analyzed by Southern blotting with the 1.5-kb *Hin*dIII fragment of pGL2 (CaMV-Chi11) containing the CaMV 35 S promoter-chitinase coding region as hybridization probe as described by Lin et al. (1995).

Hygromycin phosphotransferase (HPT) assay

Hygromycin phosphotransferase assays were done as described by Datta et al. (1990) with the modifications outlined below. The reactions were performed in a final volume of 10 μl and contained 50 μg of protein, 50 m*M* TRIS-maleate, pH 7.0, 50 m*M* $CaCl_2$, 0.05 m*M* ATP, 0.4 μl of γ-[^{32}P]-ATP (370 MBq/ml; 111 TBq/mmol) and 12.4 μ*M* of hygromycin B. Reaction mixtures without hygromycin served as negative controls. After incubation at 37°C for 1 h, 2-μl aliquots from each mixture were spotted on PEI-cellulose F TLC plates (Merck), developed in 50 m*M* sodium formate/formic acid, pH 5.4, and autoradiographed.

RNA isolation, Northern blotting, and nuclear run-on transcription

Total RNA from transgenic and control rice leaves was isolated using TRIZOL® reagent (GIBCO-BRL), and 10-μg RNA samples were loaded onto a 1.2% formaldehyde-agarose gel and electrophoresed in 1 × MOPS buffer. RNA in the gel was transferred to Gene Screen Plus™ nylon membrane (DuPont, Wilmington, Del.). The 1.1-kb chitinase coding region fragment of pGL2 (CaMV-Chi11) was used as probe. Northern blotting was carried out using standard protocols (Ausubel et al. 1987).

The nuclei isolation, run-on in vitro transcription, and hybridization were done as described by van Blokland et al. (1994). Nuclei (1×10^6) were incubated with 120 μCi of α-[^{32}P]-UTP for 1 h, and total RNA was isolated by phenol extraction. Aliquots of RNA containing 12×10^6 cpm were used in each hybridization in a final volume of 1.5 ml. The single-stranded DNA probes for detecting sense and anti-sense transcripts of rice chitinase mRNA were

prepared from appropriate M13 phages (Ausubel et al. 1987) containing the chitinase gene in the sense or antisense orientation. Aliquots (5 μg) of the DNA were immobilized on nylon membranes, cross-linked with UV, prehybridized overnight at 65°C, and then used for hybridization for 72 h. After hybridization, the filters were subjected to autoradiography.

Induction of chitinases in transgenic plants by *Rhizoctonia solani* infection

The infection of rice sheaths by the sheath blight pathogen, *Rhizoctonia solani*, was carried out as described by Ou (1985). The protein extracts (50 μg) from infected sheaths of untransformed control, "silent", and "expressing" plants were subjected to Western blot using the anti-bean chitinase antibody.

Results

Gene silencing in plants homozygous for the transgene locus

In our previous study we found that among the chitinase-positive primary transgenics, plant no. 354 had the highest level of expression compared to other transformants (Lin et al. 1995). From Southern blot and segregation analyses, we estimated that this transformant contained about five copies of the transgene that are tightly linked (Lin et al. 1995; Wang 1995). In an attempt to achieve high-level expression of chitinase transgene, we identified a T_2 generation plant, 354-7-1, that was homozygous for the transgene locus. Protein extracts of leaves from 39 progeny of 354-7-1 were analyzed for chitinase expression by Western blotting at 3 weeks through to 15 weeks after germination at 3-week intervals. All progeny contained high levels of 35-kDa and 30-kDa chitinase bands characteristic of the transgene (the 30-kDa band is presumably a truncated form of the 35-kDa band) after 3 weeks (Fig. 1A). However, some of the same plants had no detectable chitinase by 15 weeks (Fig. 1B, lanes 6 and 8) indicating that the expression of the transgene has been silenced (Fig. 1B). Out of 39 plants tested, 8 had the silent phenotype (23% of total). Once the silent phenotype was established, it was stable. Even 8 months after germination, leaf extracts from silent plants had no antibody-detectable chitinase. Sheath and root extracts from such silent plants when subjected to Western blot showed no detectable levels of chitinase. However, T_2 progeny of the low-expressing homozygous parent no. 178-2 continued to express the chitinase even at 5 months after germination and escaped silencing (data not shown).

Gene expression in plants hemizygous for the transgene locus

To determine whether the homozygous state was a requirement for gene silencing, we used Western blotting

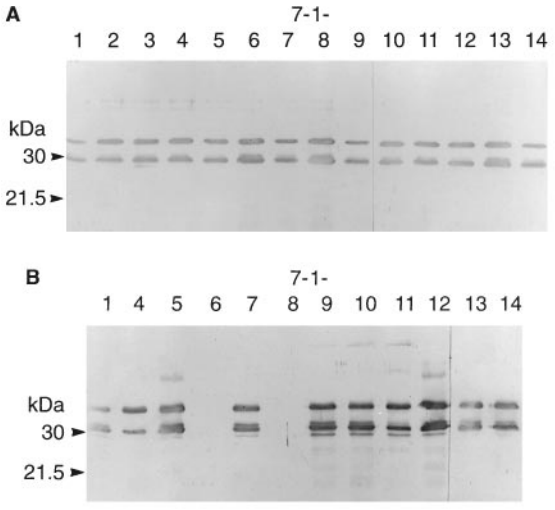

Fig. 1A, B Western blot analysis of T_3 generation plants. **A** Youngest leaves of plants nos. 1–14, progeny of homozygous parent 354-7-1, were harvested at 3 weeks after germination. Leaf extracts containing 50 μg of protein were subjected to SDS-PAGE followed by Western blotting as described in Materials and methods. Chitinases were detected using an anti-bean chitinase antibody. **B** Western blot of extracts of top leaves from the same plants at 15 weeks after germination. Each lane represents 1 plant. (Plants 2 and 3 in **A** did not survive)

to test progeny from the hemizygous parent, 354-5-5, for the presence of chitinase at different times after germination. Out of 23 progeny 8 tested negative for chitinase after 3 months. Results from 20 plants are shown in Fig. 2A. The chitinase-negative plants were tested by Southern blotting to identify those that received the transgene and those that did not. Three plants that tested negative in the Western blot assay did not have the 1.5-kb *Hin*dIII fragment diagnostic of the presence of a transgene (the other bands of sizes >1.5 kb are host chitinase bands detected by the probe), indicating that they are the chitinase gene-negative segregants. The other 5 chitinase-negative plants contained the 1.5-kb *Hin*dIII band and expressed the chitinase at 3–5 weeks, which indicated the presence of a functional chitinase transgene(s). Figure 2B shows the data from five chitinase-negative plants. Thus, even plants derived from a hemizygous parent had experienced silencing at nearly the same frequency as those derived from a homozygous parent (compare 5 out of 23 progeny of a hemizygous parent versus 8 out of 39 of a homozygous parent).

Expression of endogenous seed chitinase gene is not cosuppressed

Western blot analysis of protein extracts of seeds of non-transformed plants after SDS-PAGE detected two chitinases with sizes of 35 and 30 kDa, respectively

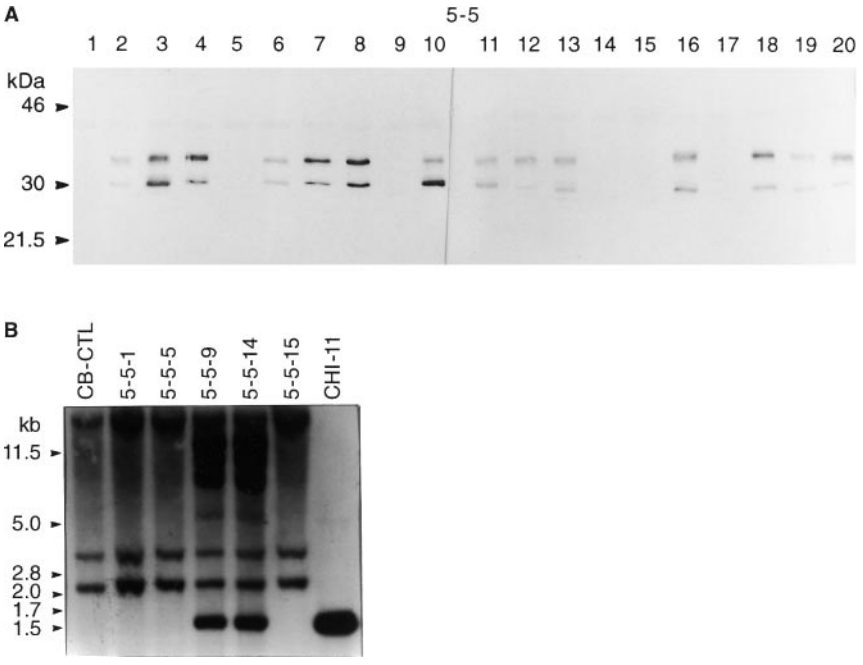

Fig. 2A, B Western blot analysis of progeny of hemizygous parent, 354-5-5. **A** Top leaves from 12-week-old plants were harvested, and extracts containing 50 μg of protein were analyzed as described in legend to **Fig. 1**. **B** Southern blot analysis of DNA from chitinase-negative plants identified in **A**. Genomic DNA (10 μg) was digested with *Hin*dIII and subjected to Southern blot analysis as described in Materials and methods. The 1.5-kb insert was used as the positive control. *Lanes CHI 11*, *CB-CTL* DNA from untransformed control plant, *lanes 2–6* DNA from plants that tested chitinase-negative in **A**

(Fig. 3). These chitinase bands had the same mobility as the ones present in leaves of transgenic plant no. 354 and were absent in control (lane CB-L) and "silent" leaves (not shown). Western blot analysis of the same extracts after native PAGE at pH 7.5 also confirmed that the two chitinase bands present in seeds had the same mobility as the two bands detected in chitinase-transgenic leaves (data not shown), indicating that the *chi11* gene is normally expressed in seeds. A comparison of the pattern of chitinase bands from Western blots of seed protein extracts after SDS-PAGE indicated that the 35-kDa and 30-kDa chitinase bands were present in control, "expressing", and "silent", plants. The levels of the chitinase bands in "silent" plants were the same as in control seeds, indicating that the expression of the endogenous host chitinase CHI11 is not affected.

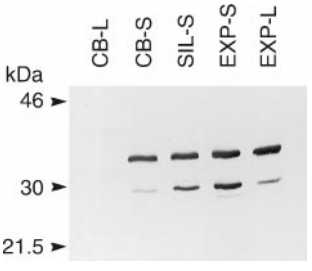

Fig. 3 Expression of endogenous seed-specific chitinase in 'silent' plants. Protein extracts from seeds (*S*) of *CB* (untransformed), *Sil* (silent), and *Ex* (expressing) plants and leaves (*L*) from *CB* and *Ex* plants were subjected to Western blot analysis as described in legend to **Fig. 1A**

Inducible leaf chitinases are not suppressed

Infection of rice sheaths (and leaves) with *R. solani* has been shown to result in the induction of two chitinases with sizes of 35 kDa and 28 kDa (Anuratha et al. 1996). To study the influence of "silencing" on the inducible chitinases, we infected rice plants with *R. solani*. Extracts of infected sheaths were analyzed by Western blotting. A comparison of the chitinase patterns of uninfected and infected sheaths from "silent" and "expressing" plants is shown in Fig. 4. Two chitinase bands of sizes 35 kDa and 28 kDa were inducible in all plants including "silent" ones, indicating that the expression of pathogen-inducible chitinases is not affected by "silencing" of the chitinase transgene.

Co-silencing of the *hpt* gene

The transgenic rice plants used in this study also contain an *hpt* gene whose expression is driven by a second CaMV 35S promoter (Lin et al. 1995). To establish whether the expression of this selectable marker gene is also subjected to silencing, we assayed extracts of leaf tissues for HPT and for chitinase. Five plants that exhibited the "silent" chitinase phenotype were chosen along with 3 "expressing" plants. Figure 5A and B shows that there was a strict correlation between the expression of the chitinase and *hpt* genes. In all 5 plants with the "silent" chitinase phenotype, the *hpt* gene was also silenced. Co-silencing of the *hpt* and chitinase genes was observed even among progeny of a hemizygous plant (data not shown).

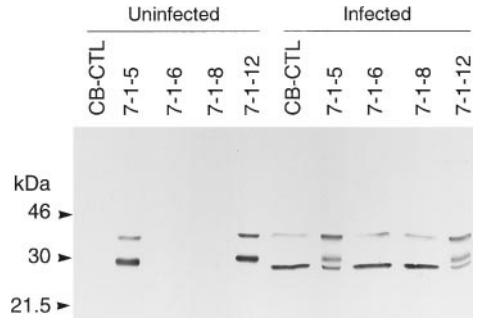

Fig. 4 Pathogen-inducible chitinase expression in 'silent' plants. Uninfected and *R. solani*-infected *CB* (untransformed), 7-1-5 and -12 (expressing), and 7-1-6 and -8 (silent) plants were subjected to Western blot analysis using chitinase antibody. Sizes of chitinase (kDa) are indicated on the left

Chitinase silencing is transcriptional

To determine whether chitinase gene silencing is transcriptional or post-transcriptional, we extracted RNA from non-transformed control, "expressing", and "silent" plants and analyzed it for chitinase transcripts using RNA blots and a chitinase probe. The results shown in Fig. 6A indicate that chitinase transcripts were detected only in "expressing" plants but not in control and "silent" plants. The absence of detectable levels of chitinase transcripts in "silent" plants may have been due to either the reduced transcription or rapid turnover of primary transcripts. A nuclear run-on transcription study was carried out to distinguish between these two possibilities. Chitinase sense transcripts (+ strand) were detected in "expressing" plants, but not in "silent" or control plants (Fig. 6B). Antisense transcripts were not detected in any samples. These results confirm that the silencing of chitinase transgenes is at the transcriptional level and not at the post-transcriptional level as has been reported in transgenic tobacco expressing a chitinase transgene (Kunz et al. 1996).

DNA from "expressing", and "silent" progeny of the homozygous parent 354-7-1 and control plants was digested with *Hin*dIII and subjected to Southern blot analysis using the 1.5-kb *Hin*dIII fragment (promoter-coding region) containing the rice chitinase gene as the hybridization probe (Lin et al. 1995). There were no differences in the pattern of autoradiographic bands between the expressing and silent plants indicating that there are no gross rearrangements or deletions of the chitinase gene (data not shown). Aliquots of DNA were also digested with *Hpa*II and *Msp*I and subjected to Southern blot analysis with either the 35 S promoter probe fragment and the chitinase coding region fragment of the transgene as probes. No differences were detected between the two DNAs (expressing and silent), suggesting that methylation of CCGG sequences can not account for the observed differences in transcriptional activity. Germination of seeds from "silent" plants in the presence of 5-azacytidine (50 mg/l) as outlined by Kumpatla et al. (1997) did not result in the reversion of the "silent" phenotype of the seedlings derived from them.

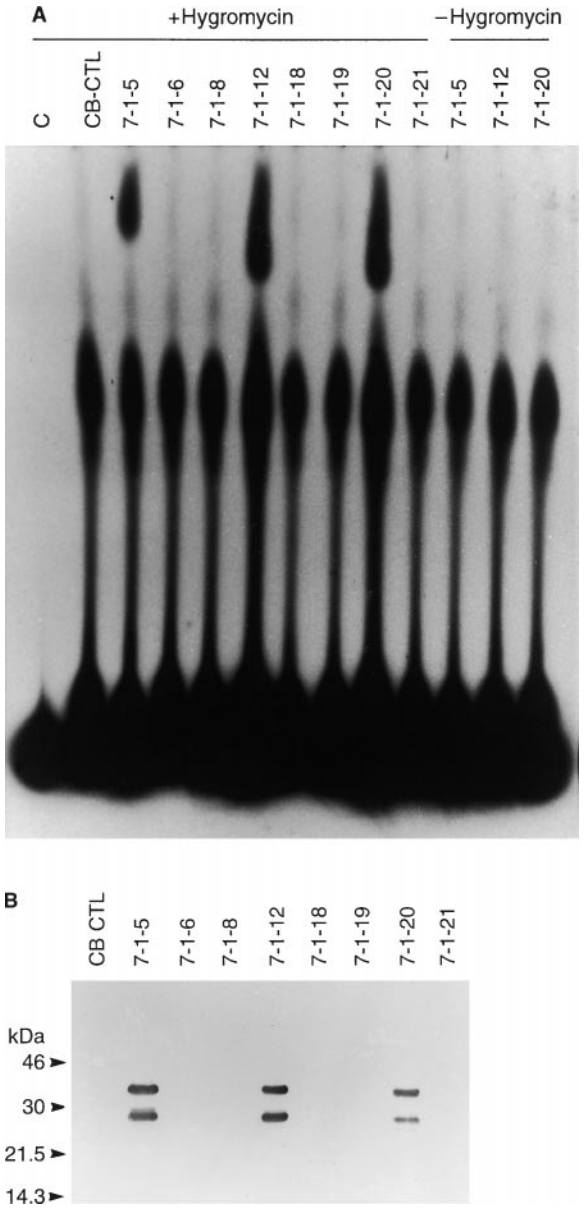

Fig. 5A, B Co-silencing of the hygromycin phosphotransferase (*hpt*) gene in plants 'silent' for transgene chitinase. **A** Leaf extracts containing 50 µg of protein were analyzed for HPT activity as described in Materials and methods. – *Hygromycin* Samples incubated without hygromycin, *CB-CTL* untransformed control, *C* enzyme-omitted control. **B** Western blot analysis of the same plants shown in **A** using chitinase antibody

Discussion

In this study we have documented the silencing of a chitinase transgene in a proportion of the progeny of

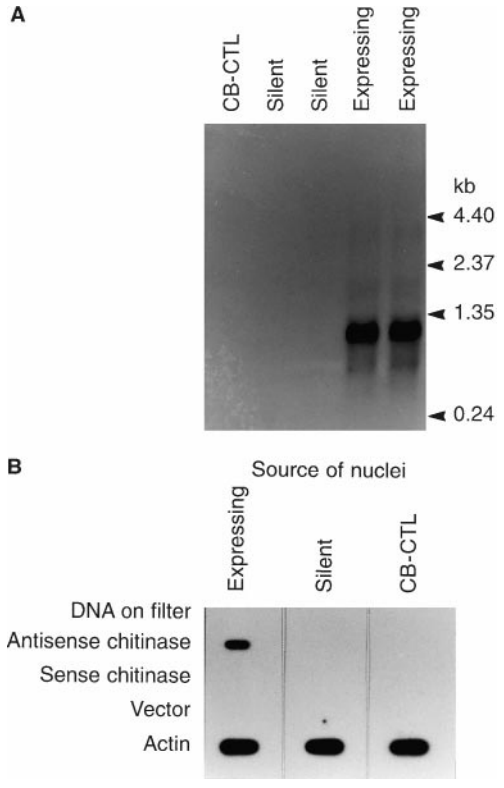

Fig. 6A Northern blot analysis of RNA isolated from untransformed (*CB-CTL*), silent, and expressing plants. Total RNA (10 μg per sample) was used and probed with the [^{32}P]-labeled 1.1-kb chitinase coding region of pGL2(CaMV-Chi11). **B** Slot blot hybridization of nuclear run-on transcription products. Single-stranded DNA (5 μg) immobilized on the membrane is indicated on the *left*. The slot blot was probed with [^{32}P]-labeled RNA isolated from the nuclei of indicated plants

a transgenic rice plant, no. 354. This plant had been shown to contain multiple copies of a rice chitinase transgene with an intact CaMV 35S promoter. The ratio of chitinase-positive to chitinase-negative plants among T_1 progeny was about 3:1, suggesting that the transgenes are tightly linked at a single locus (Lin et al. 1995). We did not observe any chitinase gene silencing in the T_0, T_1, and T_2 generations when only a limited number of progeny were analyzed. But in the T_3 progeny which were homozygous for the transgene locus, 23% exhibited the "silent" phenotype. The silencing apparently is not due to the increased copy number of the transgenes resulting from the homozygous state, because even progeny of a hemizygous parent did exhibit chitinase and *hpt* transgene silencing at nearly the same frequency as that of the progeny of homozygous plants. However, transgene silencing may be related to the level of expression of the chitinase gene. For example, homozygous progeny of another parent, no. 178, which expresses the chitinase at a five- to tenfold lower level showed no evidence of chitinase silencing. The number of copies of the chitinase transgenes in this plant (as measured by the intensity and number of bands in the *Hin*dIII digest of the genomic DNA that are unique to the transformed plant) is nearly the same as in plant no. 354 (4–5 copies of the expected size and 3–4 rearranged copies of the transgene; Lin et al. 1995). These results differ from those of Rathore et al. (1993) and Kumpatla et al. (1997) who reported that the *bar* gene was silenced only in rice plants that had several copies of the transgene but not in other transgenic lines that had relatively fewer copies of the transgene. On the other hand, Meins and Kunz (1994) observed silencing of a chitinase transgene in a transformed line that had only two to three copies of the transgene, and they favored an RNA threshold model for chitinase gene silencing in tobacco. In transgenic petunia plants, silencing was observed even in plants with a single copy of the transgene (van der Krol et al. 1990). Our results with rice chitinase transgene expression in plant no. 178 are probably due to an RNA threshold level. This line, with approximately the same transgene copy number as plant no. 354 and a five- to tenfold lower level of chitinase expression, does not experience transgene silencing. We see no correlation between copy number and silencing. Another possible explanation for the failure to observe silencing among progeny of no. 178 is that the locus of integration (for example, a genomic location close to a matrix association sequence) might have prevented it from being silenced. Allen et al. (1996) have shown that some transgenes introduced in vectors carrying matrix association sequences appear to escape silencing presumably because they end up in radial loops emerging from the matrix.

The timing of the onset of silencing in rice plants also appears to be similar to that observed in tobacco plants experiencing chitinase gene silencing where it was first seen around 6–7 weeks after germination (Kunz et al. 1996). In our studies we detected silencing at around 8 weeks. There are, however, several differences between the two systems, which suggests subtle variations in silencing mechanisms. In the transgenic rice plants, we found that while silencing could be observed in young rice leaves at around 8 weeks, by about 12–15 weeks all the leaves (including old ones) in the same plant had undetectable levels of the transgenic chitinase. Among the T_3 progeny of no. 354 that were analyzed we did not see a single mature rice plant with variable chitinase transgene expression as observed by Kunz et al. (1996) with transgenic tobacco plants. Chitinase transgene silencing was first observed in young leaves and only later in older leaves, possibly due to the time required to turn over previously accumulated chitinase. The low level of chitinase expression was shown to be a post-transcriptional effect in transgenic tobacco plants, while in progeny of rice plant no. 354 we found that silencing was due to a transcriptional block. Silencing of the chitinase transgene in tobacco was reversible and was reset during seed development

in each generation. However, in rice the silent phenotype was meiotically heritable in the subsequent generation and was maintained in the progeny while progeny of an expressing parent were prone to silencing. Kunz et al. (1996) reported that coding sequence homology rather than promoter homology was the driving force in cosuppression. We found that the *hpt* gene with the same promoter as the chitinase transgene was co-silenced, whereas the homologous host chitinase gene normally expressed in seeds was not silenced. There are other examples in the literature of cosilencing of genes that share the same promoter (Matzke et al. 1994).

The underlying mechanism of chitinase gene silencing in tobacco or rice plants is not clear. As in the case of the transgenic tobacco plants studied by Kunz et al. (1996), we failed to find any evidence of methylation of the inner C in CCGG sequences by the use of the pair of restriction enzymes *Hpa*II and *Msp*I, which differ in their sensitivity to methylation of their target CCGG sequences. However, we have not investigated the methylation of all CG and CXG sequences as was done by Park et al. (1996) in their studies with silencing of the *hpt* gene in transgenic tobacco plants. In general, silencing of genes sharing coding region homology occurs at the post-transcriptional level, whereas silencing of genes sharing promoter homology is seen at the transcriptional level (Park et al. 1996). This appears to be true for the silencing of the CaMV 35S promoter-driven rice chitinase transgene. It is pertinent to point out that neither sense nor antisense chitinase transcripts were detected in run-on transcription experiments, thereby ruling out an RNA-mediated post-transcriptional silencing mechanism. The finding that other chitinase genes whose sequences are closely related to that of the transgene are not silenced, including the *chi11* chitinase gene of the host, indicates a change in the activity of the transgene promoter. DNA sequencing of *Chi11* chitinase gene promoter regions of silent plants amplified using flanking primers by the polymerase chain reaction failed to indicate any changes in this region, ruling out the possibility that nucleotide sequence changes in the promoter caused the observed transgene silencing. The finding that the silent phenotype is passed on stably to the progeny suggests that this change is not meiotically reversible in the next generation.

The phenomenon of gene silencing may have important implications for the use of transgenic plants in combating fungal diseases. Chitinase gene silencing may make a proportion of the progeny lose their enhanced resistance conferred by the transgene(s). Environmental factors have been shown to influence the onset of gene silencing (Hart et al. 1992). The finding that silencing is observed even with a plant promoter such as the maize ubiquitin promoter (Kumpatla et al. 1997) makes it important to understand the mechanism of the onset and reversal of silencing. An in-depth understanding of the molecular basis of gene silencing in monocots will help in the management of fungal diseases by genetic engineering of cereal plants with defense genes.

Acknowledgments We thank Dr. Jianmin Zhou for his critical review of this manuscript. This work was supported by the Rockefeller Foundation. S. C. thanks the National Center for Genetic Engineering and Biotechnology, Thailand for a fellowship that supported his research at Kansas State University. The experiments reported in this paper conform to the rules and regulations of Kansas State University and regulatory agencies of the United States of America.

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