Epigenetic Transcriptional Silencing and 5-Azacytidine-Mediated Reactivation of a Complex Transgene in Rice[1]

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Despite a growing number of reports indicating non-Mendelian inheritance of transgene expression in monocots, no detailed description of the structure and stability of the transgene exists for transformants generated by direct DNA-transfer techniques, making the cause for these observations difficult to determine. In this paper we describe the complex organization of *Btt cryIIIA* and *bar* transgenes in rice (*Oryza sativa* L.) that displayed aberrant segregation in R_1 progeny. Silencing rather than rearrangement of the *bar* gene was implicated because the herbicide-sensitive R_1 plants had a DNA hybridization profile identical to that of the resistant R_0 parent and R_1 siblings. Genomic DNA analysis revealed substantial methylation of the *Ubi1/bar* sequences in silenced plants and, to a lesser degree, in herbicide-resistant plants, suggesting that the transgene locus was potentiated for silencing. Nuclease protection and nuclear run-on assays confirmed that silencing was due to transcriptional inactivation. Treatment of R_2 progeny of silenced plants with 5-azacytidine resulted in demethylation of the *Ubi1* promoter and reactivation of *bar* gene expression, demonstrating a functional relationship for methylation in gene silencing. These findings indicate that methylation-based silencing may be frequent in cereals transformed by direct DNA protocols that insert multiple, often rearranged sequences.

The advent of gene transfer has permitted novel approaches for studying gene expression and opened new avenues for the modification of crop plants. Although it is now well established that many genes introduced via *Agrobacterium tumefaciens*-mediated transformation can be expressed at high levels with correct spatial and temporal regulation, a growing number of instances have been reported in which expression has been silenced, apparently as a result of homology between introduced and resident DNA sequences (Matzke et al., 1994a). This phenomenon is much more pronounced in transgenic plants generated by direct DNA-transfer methods, which often result in multiple copies of the transgene (Klein et al., 1988; Gordon-Kamm et al., 1990; Klein et al., 1990; Rathore et al., 1993).

Homology-dependent gene silencing can occur between multiple copies of an introduced gene that are closely linked or arranged in tandem (*cis*-inactivation; Mittelsten Scheid et al., 1991; Assaad et al., 1993) or between repeated or homologous sequences at allelic (Meyer et al., 1993) or nonallelic (ectopic) chromosomal locations (*trans*-inactivation; Vaucheret, 1993; Matzke et al., 1994b). In a recent review of transgene silencing in plants, Matzke and Matzke (1995) noted that gene inactivation may result from several mechanisms, with transcriptional silencing being characteristic when promoter homology exists and posttranscriptional silencing being associated with coding sequence homology. Dorer (1997) reviewed the possibility that transgene arrays form heterochromatic regions in vertebrates, leading to repression of gene expression. McElroy and Brettel (1994) suggested that silencing may contribute to non-Mendelian segregation of transgene expression in monocots, but they also commented that no direct evidence is available in this regard.

The present study was initiated with the objective of introducing *Btt cryIIIA* encoding an insecticidal protein into rice (*Oryza sativa*) using resistance to the herbicide bialaphos (encoded by *bar*) as a selectable marker. Although both *Btt cryIIIA* and *bar* were expressed in primary transformants (R_0), expression of these genes in selfed (R_1) progeny did not exhibit the expected segregation frequency. To explore the reasons for this aberrant expression, we decided to characterize the arrangement and expression of the inserted *bar* gene, the expression of which can be readily evaluated by painting or dipping leaves in herbicide.

Three major findings became apparent. First, as is often the case in plants resulting from direct transformation approaches, the primary transformants were found to contain multiple copies of rearranged sequences in addition to a complete copy of the *bar* gene. Second, although the complex profile for the *bar* transgene in selfed progeny was identical to that of the original transformant, its presence did not follow a simple Mendelian pattern. Third, several of the R_1 seedlings that contained the complex *bar* insert were sensitive to bialaphos, indicating that its expression was silenced.

Although methylation has been shown to be associated with inactivation of gene expression in many organisms (Fedoroff, 1989; Finnegan et al., 1993; Martienssen and Richards, 1995; Matzke et al., 1996; Yoder and Bestor, 1996), this had not previously been shown for stably transformed monocot plants. Nevertheless, we surmised that it was likely to be the cause of the silencing of the *bar* transgene, and the data presented here establish that the *Ubi1* promoter (Cornejo et al., 1993) used to drive the *bar*-coding

Abbreviations: AzaC, 5-azacytidine; T309, Taipei 309.

region was extensively methylated and transcriptionally silenced in herbicide-sensitive plants. Furthermore, we were able to demonstrate loss of methylation of the *Ubi1* promoter and restoration of herbicide resistance in R_2 seedlings (derived by selfing silenced R_1 plants) when they were germinated in the presence of AzaC.

Our results not only extend the phenomenon of transgene silencing to monocots but also add to the mounting evidence that a mechanism(s) is generally present in higher plants that induces silencing of intrusive DNA (Matzke et al., 1996). As has been reported in dicots and other organisms, transgene loci containing repeat and rearranged transgene copies appear to be attractive targets for methylation. Our findings in rice, a monocot of vast importance in human nutrition, demonstrate that, even though a transgene locus containing multiple rearranged copies may be functional in primary transformants, the locus is readily methylated and frequently silenced in subsequent generations.

MATERIALS AND METHODS

Plasmids

Plasmid pJKA consists of a 1794-bp synthetic *Bacillus thuringiensis* var *tenebrionis cryIIIA* (*Btt cryIIIA*) gene fused to a cauliflower mosaic virus 35S promoter bearing an alfalfa mosaic virus translational enhancer and a nopaline synthase (*nos*) terminator (Sutton et al., 1992). The selectable marker plasmid pUbi1-bar is the same as p*Ubi-BAR* (Christensen and Quail, 1996) and contains a bialaphos-resistance gene (*bar*) under the control of a maize (*Zea mays* L.) ubiquitin (*Ubi1*) promoter and a *nos* terminator.

Biolistics-Mediated Plant Transformation

Transformation of rice (*Oryza sativa* L., sp. *japonica*, cv T309) was as described previously (Buchholz et al., 1997b). Embryos were isolated from seeds close to the end of the milky stage (approximately 2 weeks after anthesis) and subjected to bombardment (PDS 1000/He biolistics system, Bio-Rad) using pJKA and pUbi1-bar plasmids at a 5:1 (w/w) ratio on 1-μm o.d. particles (4 μg plasmid/2.4 mg particles). The day after bombardment, embryos were transferred to LS 2.5 medium (Buchholz et al., 1997b) supplemented with 4 mg/L Bialaphos (Meiji Seika Kaisha, Tokyo, Japan). Selection of resistant calli, regeneration, PCR screening of plantlets for the *Btt cryIIIA* gene, and culture of fertile plants were as detailed previously (Buchholz et al., 1997a, 1997b).

Genomic DNA Analysis

Leaf genomic DNA was isolated as described previously (Buchholz et al., 1997a). Samples (2 μg) were digested for 6 to 8 h using a 5-fold excess of the appropriate restriction enzyme, with fresh enzyme being added at 3 to 4 h, and fractionated in 0.8% agarose gels using a 1-kb DNA ladder (BRL) as a size standard. DNA was transferred to Hybond-N$^+$ membranes (Amersham), and blotting and hybridization were performed according to the manufacturers' instructions. DNA probes were generated with a

DNA-labeling kit (DECAprime II, Ambion, Austin, TX). For copy number reconstruction calculations, a 1C value (haploid DNA content of rice) of 0.5 pg (Armuganathan and Earle, 1991) was used. Plasmid DNA was digested with the same enzyme used for the digestion of transgenic plant DNA and diluted to the required copy concentration.

Bialaphos Leaf-Painting Bioassay

Transgenic seedlings and plants were tested for herbicide resistance by dipping a portion of a leaf into 0.25% (w/v) solution of a commercial herbicide (Herbiace, Meiji Seika Kaisha) containing 20% (w/w) bialaphos. The apical 8 to 10 cm of mature leaves and at least 5 cm of seedling leaves were used. Resistance to the herbicide (normal versus yellow and dried appearance) was scored after 4 to 5 d.

RNase Protection Assay

A 1807-bp *Bam*HI/*Kpn*I fragment containing a synthetic *Btt cryIIIA* gene was subcloned into pBluescript SK+ (Stratagene) to generate an antisense construct. An antisense riboprobe of 256 nucleotides (240-nucleotide *Btt cryIIIA*-coding region plus 16 nucleotides of polylinker) was synthesized by in vitro transcription using T7 polymerase on plasmid linearized with *Eco*RI. A 602-bp *Pst*I fragment containing the *bar*-coding region was subcloned into pBluescript KS+ (Stratagene). An antisense *bar* probe of 658 nucleotides (553-nucleotide *bar*-coding region plus 105 nucleotides of polylinker) was synthesized by in vitro transcription using T3 polymerase on a *Bam*HI-linearized plasmid. RNase protection assays were performed using a kit (Direct Protect Lysate RPA kit, Ambion), with 50 mg of leaf sample as the target tissue. Lysate prepared from untransformed cv T309 leaves was used as a negative control, and probe mixed with lysis buffer was used to assess the completion of RNase treatment. The protected fragments were analyzed by electrophoresis on 5% polyacrylamide/8 M urea gels using denatured, end-labeled *Hinf*I/*Dra*I fragments of pIC-20H (Marsh et al., 1984) as size standards.

Nuclear Run-On Transcription Assay

Isolation of nuclei and run-on transcription were performed essentially as described by Ingelbrecht and de Carvalho (1992). Slot blots were prepared using nitrocellulose membranes (Schleicher & Schuell) containing 1 μg of linearized plasmid DNA (or a fragment isolated from 1 μg of plasmid DNA). Hybridization was performed for 2 d and the filters were washed to a final stringency of 0.3$\times$ SSC and analyzed on a bioimaging analyzer (Fujix BAS 2000, Fuji, Tokyo, Japan).

AzaC Treatment

Reactivation of *bar* expression in the presence of the demethylating agent AzaC was tested by germinating seeds in Magenta boxes on Murashige-Skoog medium (Buchholz et al., 1997b) with (50 or 75 mg/L) or without (control) AzaC. Germination was in the dark until the

seedlings had grown to 12 to 15 cm in length, at which time they were transferred to light. Once the seedlings turned green, they were tested for herbicide resistance.

RESULTS

Generation of Transgenic Rice Plants

After co-bombardment of 38 embryos with pJKA and pUbi1-bar (see "Materials and Methods"), 3 embryos gave rise to bialaphos-resistant calli. Eleven of the 16 resulting calli yielded at least 1 bialaphos-resistant plant each. Ten plants, designated JKA 50, 51, 52, 53, 54, 56, 58, 59, 60, and 61, yielded the expected product when subjected to PCR analysis using primers specific to the *Btt cryIIIA*-coding region and were transferred to soil. Although all plants derived from herbicide-selected callus should be bialaphos resistant, past experience has shown that plants chimeric for herbicide resistance can result from biolistic transformation (S. P. Kumpatla, W. G. Buchholz, and T. C. Hall, unpublished results). To check for chimerism, at least one leaf of each tiller of plants was tested for herbicide resistance before and after the maximum tillering. All tillers from all plants were found to be resistant, reflecting the presence and expression of the *bar* gene and indicating the absence of chimerism. These primary (R_0) transgenic plants were morphologically similar to wild-type cv T309 plants with respect to vegetative growth, flowering behavior, and seed set.

Genomic DNA Analysis of Primary Transformants

Direct DNA uptake-mediated transformation often leads to production of transgenic plants containing multiple copies of the introduced gene, many of which are fragmented or rearranged. Because of this we believed that it was crucial to analyze the structural integrity of the transgene and its regulatory elements before subjecting the plants to further experimentation and progeny testing. Since our primary objective was the generation of transgenic rice plants that contain and stably express the *Btt cryIIIA* insecticidal gene, extensive molecular analyses were carried out to assess the integrity and organization of the *Btt cryIIIA* gene in R_0 transgenic plants.

A map of pJKA, probes used for genomic DNA analyses and RNase protection assays, and the expected fragments are shown in Figure 1A. Initial genomic DNA blots of the 10 primary transformants revealed three distinct hybridization patterns when the *Btt cryIIIA*-coding region fragment was used as a probe, suggesting that they were derived from three independent transformation events (which is consistent with three different bombardments used to generate these plants). On the basis of these hybridization patterns, the plants were designated group I (JKA 50, 51, 52, 53, and 54), group II (JKA 56 and 58), and group III (JKA 59, 60, and 61).

Genomic DNA was digested with *Eco*RV restriction endonuclease that releases a 1634-bp fragment spanning the 3' region of the cauliflower mosaic virus 35S promoter and most of the *Btt cryIIIA*-coding region. When the blot was
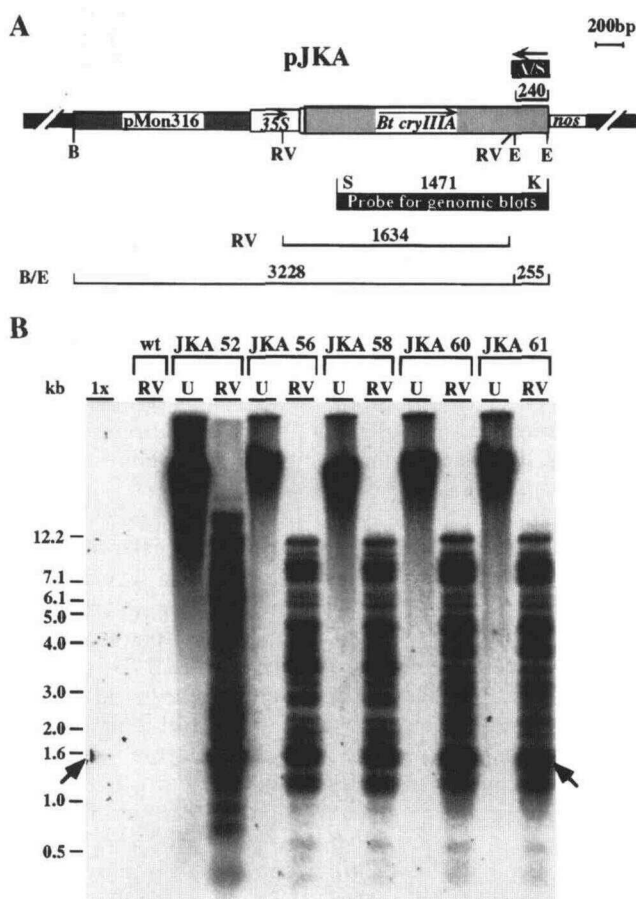

Figure 1. Genomic DNA analysis of R_0 transformants. A, Proportional map of pJKA showing functional regions and restriction sites used for DNA analysis. The hybridization probe and the expected fragments are shown below the map. The location of the antisense probe (A/S) for RNase protection assays and the predicted 240-nucleotide protected fragment are shown at the top. B, Genomic DNA analysis. Genomic DNA was digested with *Eco*RV or undigested and hybridized with the indicated 1471-bp probe corresponding to most of the *Btt cryIIIA*-coding region. B, *Bam*HI; E, *Eco*RI; K, *Kpn*I; RV, *Eco*RV; S, *Sac*I; U, undigested; 1x, 1-copy reconstruction of pJKA DNA; and wt, untransformed cv T309. Arrows indicate locations of the expected fragments.

hybridized with the indicated 1471-bp *Sac*I/*Kpn*I probe, the expected fragment (with an intensity equivalent to more than 10 copies based on a comparison with 1-copy reconstruction) was observed in all lanes (Fig. 1B). In addition, many bands larger and smaller than the expected fragment were also detected in each line, apparently due to fragmentation and rearrangement of the input DNA. It was also apparent that the transgenic line JKA 52 contains a higher level of input DNA compared with JKA 56, 58, 60, and 61, as evidenced by the strong hybridization intensities of all of the observed bands. In all cases the *Btt cryIIIA* gene was integrated into the genome, which was indicated by its strong hybridization to high-molecular-weight DNA (Fig. 1B, lanes U).

To determine whether a full-length *Btt cryIIIA* gene was present and to determine the integration pattern differ-

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ences of the three groups, genomic DNA from JKA 52, 58, and 60 (representing groups I, II, and III, respectively), was digested with BamHI/EcoRI or KpnI and hybridized with the SacI/KpnI probe corresponding to the Btt cryIIIA-coding region (Fig. 2A). BamHI/EcoRI hybridization bands corresponding to the expected 3228- and 255-bp fragments (which contain the 35S promoter and the Btt cryIIIA-coding region; see Fig. 1A) were present in all three lines. A comparison with copy reconstruction revealed that JKA 58 and 60 contained four to five copies of the full-length sequence, and JKA 52 had about double that number. Hybridization patterns of the KpnI digests clearly indicate three distinct profiles, supporting their classification into three groups (corresponding to three integration events). The existence of multiple bands further confirms the previous results that fragmentation and rearrangement of the input DNA appear to be common.

RNase protection assays to detect the presence of Btt cryIIIA mRNA in JKA 52, 58, and 60 revealed that Btt cryIIIA transcripts were present only in line JKA 52 (data not shown). Although JKA 58 and 60 have an intact Btt cryIIIA gene, they do not display the corresponding mRNA, suggesting that the Btt cryIIIA in JKA 58 and 60 is either silenced or that the fragments observed at the expected location may not represent full-length, non-rearranged copies. Tests for rice water weevil resistance were conducted using line JKA 52 because it was the only line that expressed the Btt cryIIIA gene. However, when R_1 seedlings from the JKA 52 line were subjected to rice water weevil feeding bioassays, protection ranged from complete (all larvae were dead) to that of wild-type plants (no protection). Since only a low level of Btt cryIIIA mRNA was detected in the R_0 plant, it seemed possible that the heterogeneous resistance reflected variable transcript levels.

To examine this possibility, RNase protection assays were conducted for 33 R_1 plants (Fig. 2B shows the data for 21 plants). It was surprising that Btt cryIIIA mRNA was present in only 7 plants, indicating aberrant segregation (24 plants were expected, since the transgene is present at a single locus) or transgene silencing. It was also interesting to note that in the parent line JKA 52 there were two protected bands (a 240-nucleotide expected band and a shorter band of about 225 nucleotides). The mRNA corresponding to the smaller band probably resulted from transcription of a truncated version of the Btt cryIIIA gene due to the rearrangements. However, in those progeny lines showing expression of Btt cryIIIA, the shorter band was very faint or absent, suggesting low or no detectable steady-state levels of this transcript, respectively.

To conveniently evaluate a large number of progeny plants for transgene expression to understand the basis of the observed lack of expression in several progeny plants, the bar gene was chosen for further analysis since its expression can be readily assayed by testing the leaves for herbicide resistance or sensitivity. Progeny were obtained by selfing JKA 52. Of 108 seedlings that were tested for bialaphos resistance, 53 were resistant and 55 were sensitive (a 1:1 ratio), indicating that the bar gene also segregated aberrantly in this transgenic line.

R_0 Transgenic Plants Contain Multiple Rearranged Copies of bar

A map showing the pUbi1-bar construct, probes used for genomic DNA analyses, and the fragments expected to be present following digestion with the indicated restriction endonucleases is shown in Figure 3. Genomic analysis of the 10 primary transformants revealed three distinct hy-
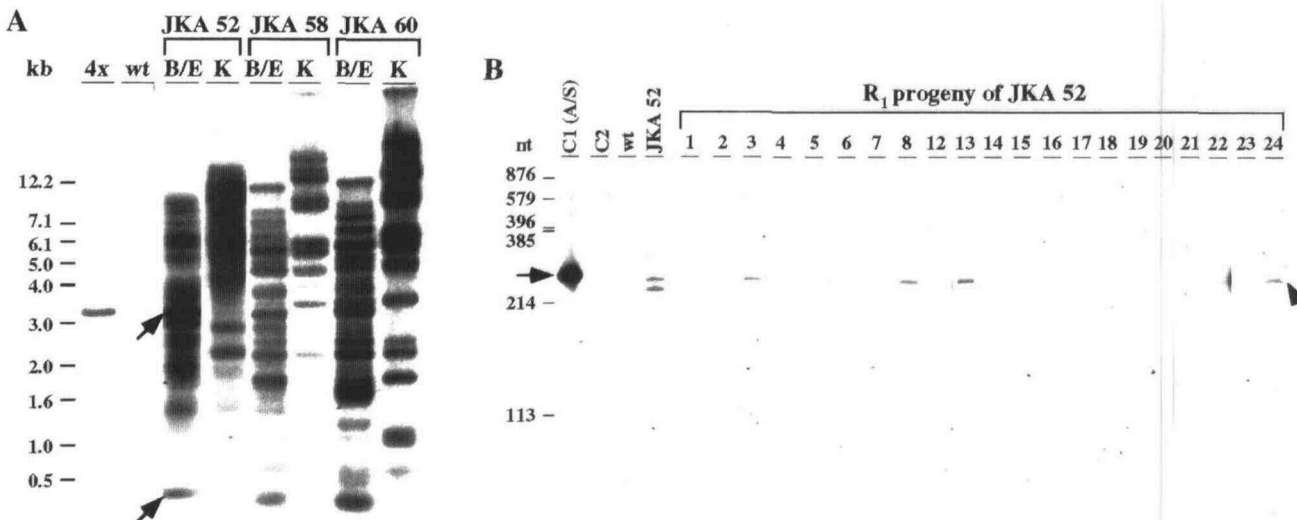

Figure 2. Genomic DNA analysis of R_0 transgenic plants and RNase protection assays of R_1 progeny of JKA 52 line. A, Genomic DNA (2 µg; 1 µg in the case of JKA 52) was digested with BamHI/EcoRI (B/E) or KpnI (K) and hybridized with the SacI/KpnI probe indicated in Figure 1A. 4x, Four-copy reconstruction of pJKA; wt, untransformed cv T309. The expected fragments are indicated by arrows. B, Leaf lysates from JKA 52 (R_0) and 21 progeny plants (R_1) were hybridized with a 256-nucleotide Btt cryIIIA antisense probe (C1 [A/S]; see Fig. 1A for location of the probe) and digested with RNase. C2, Control containing buffer, probe, and RNase; wt, control containing lysate from untransformed cv T309, probe, and RNase. Molecular sizes are indicated on the left; the probe and the expected fragments are indicated by arrows.
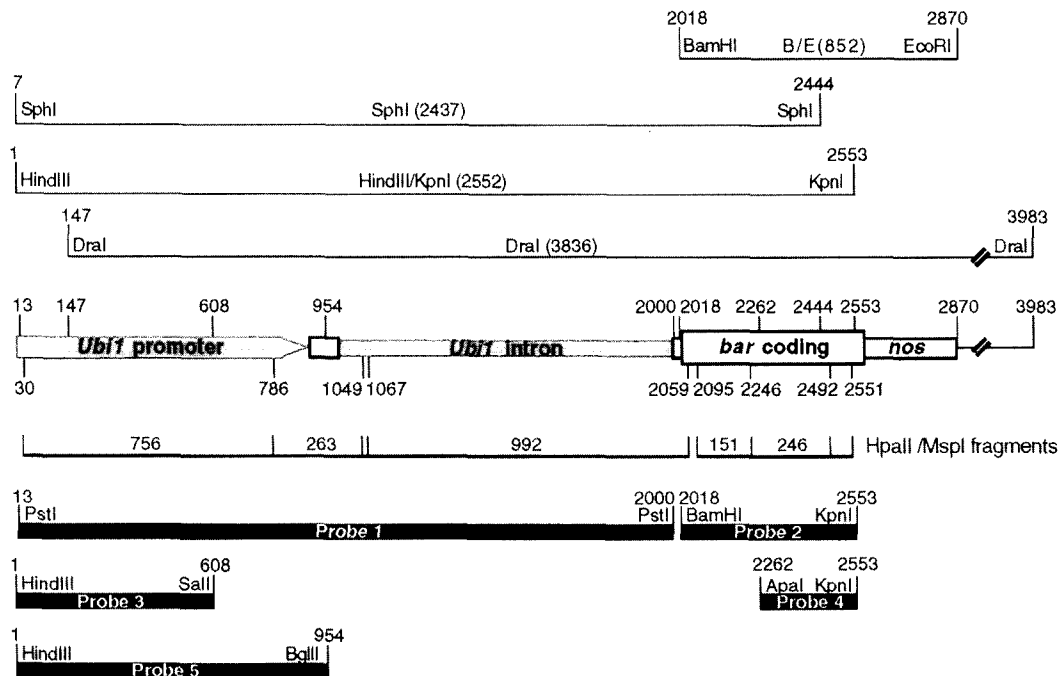
Figure 3. Proportional map of pUbi1-bar showing functional regions. Restriction sites used for genomic analysis and expected fragments are shown at the top, and locations of HpaII/MspI sites and expected fragments are shown below. Solid bars denote radioactive fragments used as hybridization probes.

bridization patterns when the bar-coding region was used as a probe (data not shown), suggesting that they were derived from three independent transformation events. When the Btt cryIIIA-coding region was used as a probe the plants fell into the same three groups (Figs. 1B and 2A).

Since the transformants contained multiple copies of Btt cryIIIA, it is expected that the bar gene also represents a similar situation. One method to determine whether a full-length, intact copy of the bar gene was present in these plants was to ascertain whether individual fragments that span the gene were physically present. For instance, to determine whether the Ubi1 promoter was present, rice genomic DNA was digested with SphI and hybridized with a corresponding probe (Fig. 3, probe 1). A band corresponding to the expected 2437-bp SphI fragment with a hybridization signal equivalent to a single, intact copy of the Ubi1 promoter was observed for JKA 52 (Fig. 4A). A band migrating at approximately the same position was present for the other lines, but its weaker hybridization suggested that it might represent a rearranged fragment (confirmed below). Several additional fragments were detected in each line, presumably representing fragmentation or rearrangement of the input DNA.

Further genomic analysis using a bar-coding region-specific probe (Fig. 3, probe 2) showed that JKA 52 contained the expected 2552-bp HindIII/KpnI fragment (Fig. 4B) and a 852-bp BamHI/EcoRI fragment (Fig. 4C). Comparison of the hybridization intensity with that of the reconstruction in both cases indicated that two intact copies of the expected fragments were present in JKA 52. To confirm that the HindIII/KpnI fragment in JKA 52 was an intact copy of the promoter and coding region of the se-

lectable marker gene, this blot was stripped and rehybridized with a 607-bp HindIII/SalI probe (Fig. 3, probe 3) that spanned the extreme 5' region of the Ubi1 promoter. The blot was subsequently stripped and rehybridized with a 291-bp ApaI/KpnI probe (Fig. 3, probe 4) corresponding to most of the 3' portion of the bar-coding region (data not shown). In both cases hybridization to the expected 2552-bp HindIII/KpnI fragment was detected, confirming the contiguity of the promoter and bar-coding region. In addition, hybridization with probes 3 and 4 also revealed the promoter- and coding-region composition of all of the HindIII/KpnI fragments (Fig. 4B, fragments denoted P and C). From these data, it is apparent that the JKA 52 line contained two copies of promoter sequence with >600 bp of homology to the full-length promoter and several smaller promoter fragments (denoted as P and p, respectively, in Fig. 4B).

Genomic analysis of transgenic lines JKA 58 and 60 indicated the absence of the expected 2552-bp HindIII/KpnI fragment (Fig. 4B). The possibility that this was due to methylation was ruled out by using the methylation-insensitive enzymes AflII, DraI, and ScaI, either by themselves or in combination with HindIII or KpnI (data not shown). Further analyses indicated that these plants lack the DraI/KpnI fragment but contain the BamHI/EcoRI fragment and all of the other predicted fragments between the SalI[608] site and the EcoRI[2870] site (Fig. 4C and data not shown). Since these plants were bialaphos resistant, these data suggest that disruption occurred in the upstream approximately 600 bp of the Ubi1 promoter and that the downstream approximately 300 bp are adequate to drive expression of the bar-coding region. This analysis con-
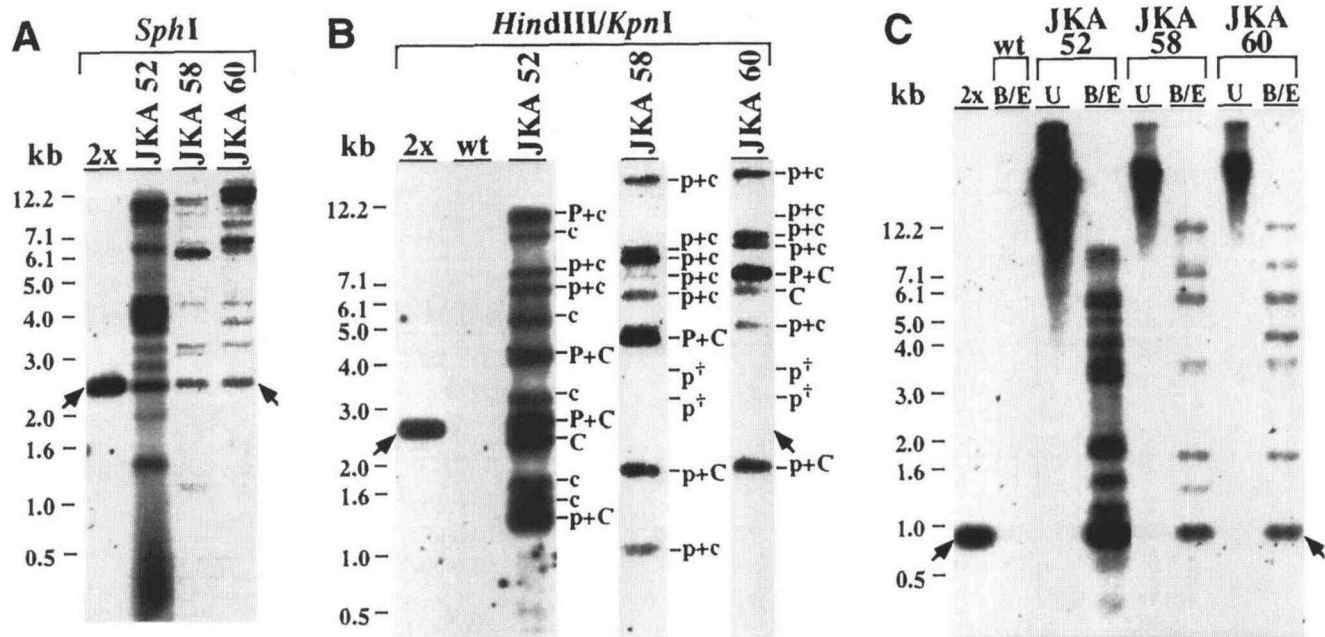
Figure 4. Genomic DNA analysis of primary transformants. Genomic DNA was digested with *Sph*I (A), *Hin*dIII and *Kpn*I (B), or *Bam*HI and *Eco*RI (B/E) or undigested (U) (C). Probe 1 (see Fig. 3 for details of probes) was used for hybridization in A and probe 2 was used for hybridization in B and C. The blot shown in B was sequentially stripped and rehybridized with probes 3 (promoter) and 4 (*bar*-coding region). P and C, Fragments hybridized to probes 3 and 4, respectively (P/C, strong signal; p/c, weak signal). p⁺, Positions of fragments that hybridized only to probe 3; wt, untransformed cv T309; 2x, two-copy reconstruction of pUbi1-bar DNA. Arrows indicate locations of expected fragments.

firmed that the weakly hybridizing *Sph*I fragment of approximately 2437 bp in these lines (Fig. 4A) was fortuitous and does not correspond to the intact promoter fragment present in JKA 52.

These findings also suggest that bialaphos resistance was conferred by the P plus C fragments (approximately 4.4 kb for line JKA 58 and approximately 7.2 kb for JKA 60) shown in Figure 4B, but they do not exclude the possibility that resistance was bestowed by another *Hin*dIII/*Kpn*I fragment (e.g. one of the p plus C or p plus c fragments shown in Fig. 4B). In all cases, the selectable marker was integrated into the genome, because strong hybridization to high-molecular-weight DNA was observed when a genomic DNA blot containing undigested DNA from JKA 52, 58, and 60 was hybridized to a *bar*-coding-region probe (Fig. 4C).

Evidence for Silencing of the *bar* Gene in JKA 52 R₁ Progeny Plants

A random sample of 20 JKA 52 progeny plants was subjected to genomic DNA analysis (Fig. 5 shows the data for 12 plants) to evaluate whether the *bar* gene might have been inactivated because of DNA rearrangement(s). Ten of the randomly selected progeny plants contained the *bar* gene. As expected, those that lacked the gene were sensitive to bialaphos and most that had the gene were resistant. However, three of the plants that had the gene (plants 52–9, 52–10, and 52–15) were sensitive to bialaphos.

Leaves that were resistant were totally green and the leaves from the sensitive lines were yellowish and shriveled throughout the painted area. We did not observe any partial or mosaic kind of phenotype with respect to herbicide resistance. Careful examination substantiated that they had the same DNA fragment profile as the parent and the bialaphos-resistant progeny, indicating a lack of detectable rearrangement in the promoter and coding region of the *bar* gene.

To determine whether the terminator might have been rearranged in these three plants, the DNA was digested with *Dra*I (which spans most of the promoter, the coding region, the terminator, and 1.1-kb vector sequence downstream of the gene) or *Bam*HI/*Eco*RI (which releases a fragment containing the coding region and terminator). After hybridization with the *bar*-coding region (Fig. 3, probe 2), the patterns of the silenced plants were identical to those of the resistant plants (Fig. 6), indicating a lack of gross rearrangement in the terminator region. Taken together, these data strongly suggest that the *bar* gene was physically intact but silenced in these three progeny plants. If the chromosomal position of the gene insert were a primary determinant in the observed silencing, it would seem logical that all or most of the progeny plants should exhibit silencing. Since only 15% of the seedlings tested were silenced, it appears that some factor other than position effect activates the silencing response.

In most cases of co-transformation, both genes are integrated into the same genetic locus. Therefore, those prog-
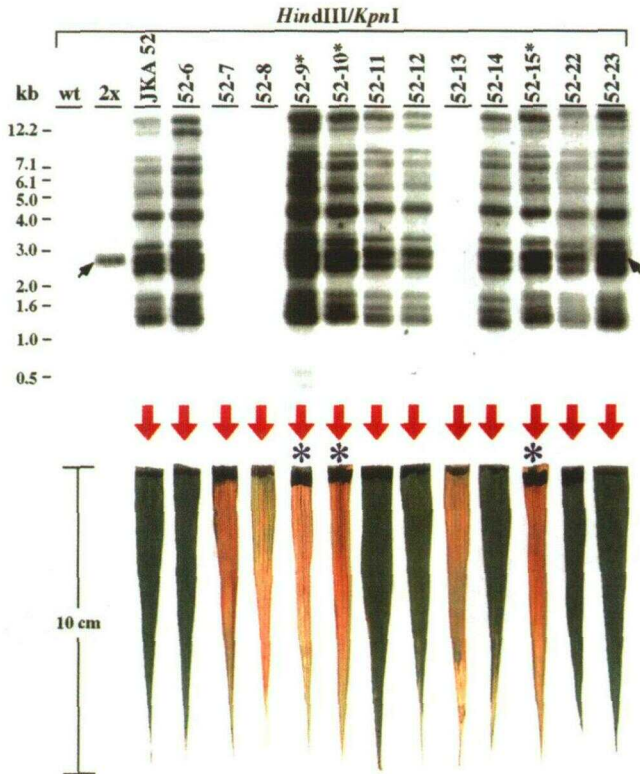
Figure 5. Genomic analysis and herbicide (bialaphos) leaf-painting bioassays of JKA 52 progeny. Top, Genomic DNA from JKA 52 and 12 R_1 plants was digested with HindIII and KpnI and hybridized with probe 2 (see Fig. 3). wt, Untransformed cv T309; 2x, two-copy reconstruction of pUbi1-bar DNA. The expected 2552-bp fragment containing the Ubi1 promoter and the bar-coding region is indicated by an arrow. Bottom, Bialaphos leaf-painting results for the progeny plants. Leaf portions of 8 to 10 cm were marked and tested for bialaphos resistance by dipping in herbicide solution (see "Materials and Methods"). Green areas reflect resistance to bialaphos; yellowish and shriveled appearance indicates sensitivity. Asterisk (*) denotes that bar is physically present but silenced.

eny that lack the bar gene should also lack the Btt cryIIIA gene. The genomic blot shown in Figure 5 was stripped and rehybridized with a probe made from vector sequences common to both chimeric gene plasmids (data not shown). Hybridization was detected to DNA from only those plants that had hybridized to the bar probe, indicating that the plants recessive for bar are also recessive for the Btt cryIIIA gene and multiple vector backbone sequences, commensurate with integration at the same locus. The parent and all of the transgene-containing progeny gave a similar, if not identical, hybridization pattern, which is also consistent with integration at a single locus.

Analysis of progeny from the JKA 58 and JKA 60 transformants revealed 3:1 and 15:1 segregation, respectively, for herbicide resistance. These ratios are commensurate with insertion at one and two loci, respectively. We found no evidence for silencing in the parental plants (JKA 58 and 60) or their first-generation progeny. Compared with JKA 52, these two lines had a much simpler pattern for the inserted bar sequences and only one fragment had a

near full-length promoter (denoted by P in Fig. 4B). These findings support the speculation that silencing in line JKA 52 results from the multiplicity of inserted, rearranged sequences.

Cytosine Methylation Is Associated with Silencing of the *bar* Gene

Cytosine methylation has been correlated with many instances of gene silencing in dicotyledonous plants (Finnegan et al., 1993; Finnegan and McElroy, 1994; Flavell, 1994). Therefore, the methylation status of the bar gene in bialaphos-resistant and bialaphos-sensitive plants was investigated by genomic analysis using the restriction endonuclease isoschizomers HpaII and MspI (Fig. 7). Both enzymes recognize the sequence CCGG, but HpaII cleaves DNA only when both cytosines are unmethylated, whereas MspI cleaves both the unmethylated sequence and the sequence in which the internal cytosine is methylated (C^m-CGG).

When promoter-specific probe 5 was used, HpaII fragments of the predicted sizes (756 and 263 bp) were detected in the herbicide-resistant plants JKA 52 (R_0) and 52–6 (R_1) but not in the sensitive plants JKA 52–9, 52–10, and 52–15 (Fig. 7A). That the different DNA fragment profiles reflect methylation, rather than a loss of the restriction recognition site, was confirmed by using MspI, which released the
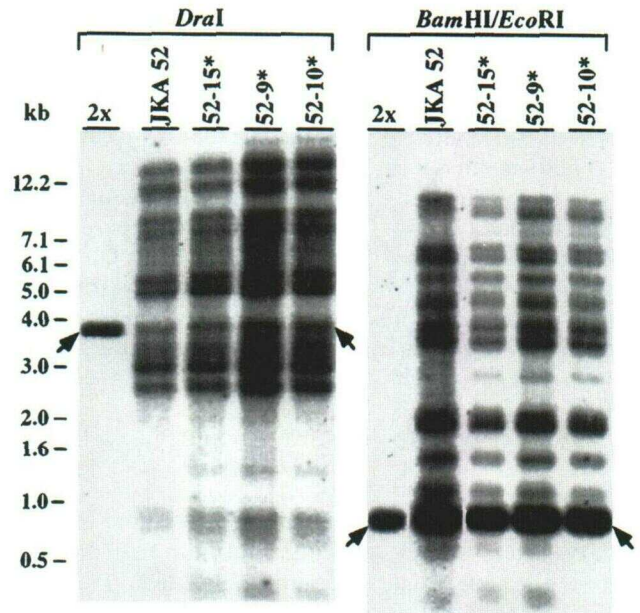

Figure 6. Genomic analysis of silenced lines to confirm the physical integrity of Ubi1 promoter, bar gene, and terminator. Genomic DNA from herbicide-sensitive (52–9, 52–10, and 52–15) progeny and the parent JKA 52 was digested with DraI (left) or BamHI/EcoRI (right) and hybridized with probe 2 (see Fig. 3; probe 2). The expected 3836-bp fragment in the left panel harbors the Ubi1 promoter, bar-coding region, nos terminator, and vector sequences, and the expected 852-bp fragment in the right panel encompasses the bar-coding region and nos terminator (see Fig. 3); 2x, two-copy reconstruction of pUbi1-bar DNA. Asterisk (*) indicates a bialaphos-sensitive plant.
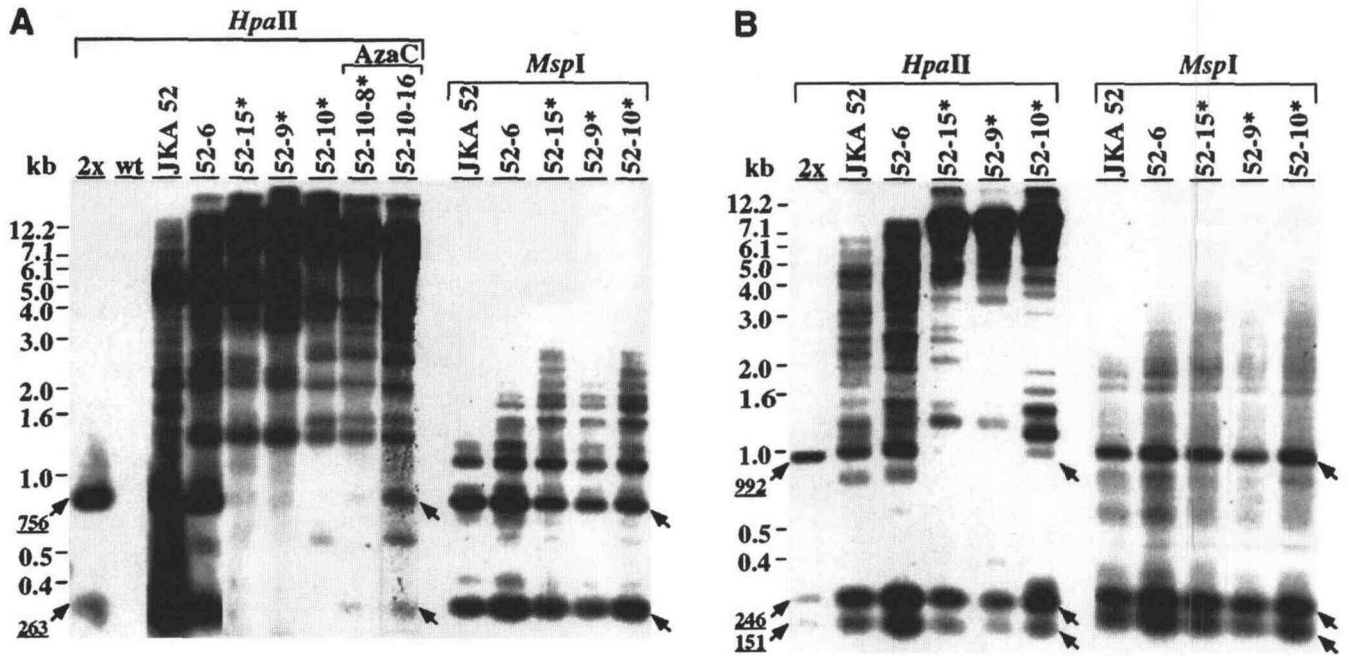
Figure 7. Methylation status of DNA in silenced and reactivated plants. Genomic DNA was digested with either *Hpa*II or *Msp*I. A, Blots were hybridized with promoter-specific probe 5 (see Fig. 3). Note the absence of the 756- and 263-bp fragments in the DNA of bialaphos-sensitive plants digested with *Hpa*II and the presence of these fragments when DNA was digested with *Msp*I. These fragments were partially (52–10–8) or fully (52–10–16) restored in R_2 seedlings grown in the presence of AzaC. B, Blots were hybridized with probe 2, corresponding to the coding region. Note the increased amounts of high-molecular-weight fragments and the greatly diminished 992-bp fragment in the bialaphos-sensitive plant DNA digested with *Hpa*II. wt, DNA from untransformed cv T309; 2x, two-copy reconstruction of pUbi1-bar DNA. Asterisk (*) indicates a bialaphos-sensitive plant; arrows indicate locations of expected fragments.

expected fragments from both bialaphos-resistant and bialaphos-sensitive plants. Indeed, the general upward shift in molecular weight of many *Hpa*II fragments in the profile obtained for the sensitive plants (compared with that for resistant plants) indicates that their DNA had undergone extensive methylation. At least partial cytosinemethylation is also apparent in resistant progeny plant 52–6, because its hybridization pattern differs subtly from that of the parent plant (compare lanes JKA 52 and 52–6 in Fig. 7A).

Using the coding-region probe, we found that the 992-bp *Hpa*II fragment that spans the *Ubi1* intron was present in resistant plants JKA 52 and 52–6, whereas it was not detectable (52–9) or only barely detectable (52–10 and 52–15) in the sensitive plants (Fig. 7B), indicating that the transcribed but untranslated region (*Ubi1* intron 1) is also methylated in these plants. In contrast, the predicted 246- and 151-bp *Hpa*II fragments were present in both the resistant and sensitive plants, indicating the presence in each of at least one unmethylated copy of the coding region. However, methylation of some *bar*-coding region fragments was evident and, overall, the bialaphos-sensitive plants contained more high-molecular-weight hybridizing DNA, indicating a higher degree of methylation in these plants than in the bialaphos-resistant plants (Fig. 7B). Taken together, these data suggest that cytosine methylation is involved in silencing expression of the *bar* gene and, specifically, that

silencing is primarily due to methylation of the promoter and the 5' untranslated region. In fact, comparison of the *Hpa*II or *Msp*I patterns obtained using either the promoter- or coding-region probes revealed that the inserted locus was partially methylated, even in JKA 52, the herbicide-resistant parent (R_0) plant (Fig. 7).

Evidence That Silencing of *bar* Is at the Level of Transcription

To investigate the status of *bar* gene transcription in resistant and silenced plants, we undertook both RNase protection assays (as a very sensitive method to detect the presence of mRNA) and nuclear run-on assays (to determine the dynamic status of transcription). Hybridization of total plant mRNA to a 658-nucleotide *bar* antisense probe followed by RNase digestion yielded a protected fragment of the predicted length, 553 nucleotides (which spans the *bar*-coding region) for bialaphos-resistant but not for bialaphos-sensitive plants (Fig. 8A). Three protected fragments were faintly visible in all lanes that contained plant mRNA, including the wild-type control, and were probably due to cross-hybridization to a related plant mRNA. Other less than full-length fragments were present only in the lanes containing mRNA from resistant plants and probably corresponded to truncated transcripts from rear-
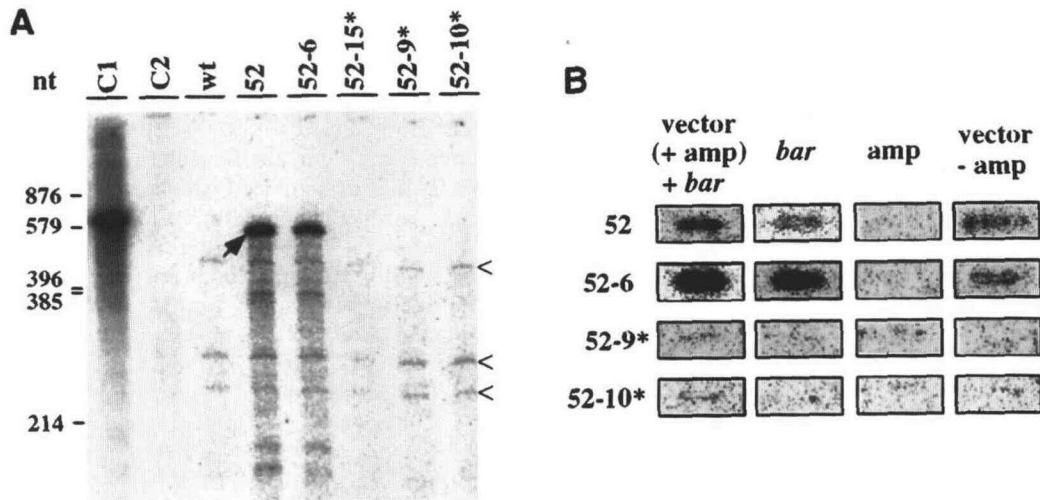
Figure 8. Evidence that silencing operates at the level of transcription. A, RNase protection assay. Leaf lysates from bialaphos-resistant, -sensitive, and untransformed cv T309 (wt) were hybridized with a 658-nucleotide *bar* antisense probe (C1) and digested with RNase. Bialaphos-resistant parent (JKA 52) and R_1 (52–6) plants show a protected band of the expected size (553 nucleotides), indicating the presence of *bar* mRNA; this band is absent in bialaphos-sensitive plants. C2, Control containing buffer, probe, and RNase. Positions for size markers are shown on the left. Bands resulting from cross-hybridization to an endogenous mRNA are marked (<). B, Nuclear run-on transcription analysis. [^{32}P]UTP-labeled run-on transcripts prepared from herbicide-resistant (JKA 52 and 52–6) and sensitive (52–9 and 52–10) plants were hybridized to slot blots containing the indicated double-stranded DNA samples: *bar*, *bar*-coding fragment only; *amp*, ampicillin fragment from vector; and vector-*amp*, vector without ampicillin fragment. Asterisk (*) indicates a bialaphos-sensitive plant.

ranged copies of the *bar* gene or possibly to partially degraded mRNA.

That silencing resulted from transcriptional inactivation was confirmed by nuclear run-on assays. Active transcription of the *bar* gene was detected for the herbicide-resistant parental plant JKA 52 and progeny plant 52–6, but only background levels of hybridization were detected for the silenced progeny plants 52–9 and 52–10 (Fig. 8B). Weak hybridization to vector fragments was also detected in JKA 52 and 52–6. This probably reflects transcription of fragmented vector sequences, since only background signal was obtained in the ampicillin region of the vector (which is farthest from the site of insertion of the *Ubi1/bar* sequence in the vector used for cloning and biolistic transformation). The fact that transcription of both *bar* and vector sequences was lacking in progeny plants 52–9 and 52–10 is consistent with methylation and silencing of the

Ubi1 promoter (and also of the 35S promoter driving *Btt cryIIIA*) throughout the inserted locus.

Reactivation of *bar* Gene Expression by AzaC Treatment

To further support our belief that transcriptional silencing of the *bar* gene resulted from cytosine methylation, R_2 progeny of the silenced plants were subjected to AzaC treatment. This cytidine analog integrates into DNA during replication or repair and prevents methylation by inhibiting DNA methyl transferase (Santi et al., 1983). Seeds collected from the silenced R_1 plants 52–9, 52–10, and 52–15 were germinated in the absence or presence of 50 or 75 mg/L AzaC (Table I). Seeds from the resistant plant 52–6 were germinated without AzaC as a control and segregated in a 3:1 ratio for bialaphos resistance. Although this ratio is expected for stable Mendelian inheritance of a selfed het-

Table I. *Reactivation of bar gene expression in R_2 seedlings by AzaC treatment*

R_1 Plant No.	Control (No AzaC)			50 mg/L AzaC			75 mg/L AzaC		
	Resistant	Sensitive	Total no. tested	Resistant	Sensitive	Total no. tested	Resistant	Sensitive	Total no. tested
				No. of plants					
52–6[a]	35 (76%)	11 (24%)	46	ND[b]	ND	ND	ND	ND	ND
52–9*[c]	0	41	41	8 (24%)	26 (76%)	34	15 (58%)	11 (42%)	26
52–10*	0	42	42	12 (29%)	30 (71%)	42	19 (70%)	8 (30%)	27
52–15*	0	41	41	9 (25%)	27 (75%)	36	ND	ND	ND

[a] Plant 52–6 contained a functional *bar* gene.   [b] ND, Not determined.   [c] Asterisk (*) indicates plants containing an intact but silenced *bar* gene.

erozygote, further genetic analysis of progeny revealed that plant 52–6, and indeed all silenced R_1 plants, were in fact homozygous. Therefore, the sensitive R_2 seedlings from 52–6 resulted from silencing.

Without AzaC treatment, all of the R_2 seedlings from the bialaphos-sensitive R_1 plants 52–9, 52–10, and 52–15 were sensitive to bialaphos. In contrast, after AzaC treatment 24 to 70% of the R_2 seedlings were resistant to herbicide, indicating reactivation of the *bar* gene. It is possible that a further increase in concentrations of AzaC or different methods of application and herbicide testing would increase the proportion of reactivated seedlings.

The herbicide painting results from the AzaC experiment are strictly bimodal, i.e. leaves from reactivated lines were green throughout the painted area, whereas those from nonreactivated plants were yellowish and shriveled, without any mosaic pattern. We have followed several reactivated lines for the retention of the restored *bar* gene expression and observed uniform resistance throughout the leaf samples tested without any spotty appearance, whereas the nonreactivated lines maintained a yellowish phenotype (S.P. Kumpatla and T.C. Hall, unpublished data).

The methylation status of the promoter region of the *Ubi1/bar* gene was determined for several R_2 seedlings. *Hpa*II fragments of 756 and 263 bp, which were absent in the bialaphos-sensitive R_1 plants, were also absent in the sensitive R_2 seedlings. However, these two fragments were detected in reactivated seedlings exhibiting bialaphos resistance (a representative, 52–10–16, is shown in Fig. 7A), thereby directly correlating loss of methylation with activation of the *Ubi1* promoter. Partial demethylation was also observed in some seedlings, e.g. in seedling 52–10–8 (Fig. 7A), which was apparently herbicide-sensitive, probably accounting for the non-Mendelian segregation of herbicide resistance after AzaC treatment. As explained above, the reactivated line 52–10–16 displayed a totally green phenotype and the nonreactivated line 52–10–8 displayed a uniform yellowish phenotype throughout the leaf area painted.

DISCUSSION

Transgene Silencing and Methylation

Although instability of transgene expression has been observed in maize (Klein et al., 1990) and rice (Register et al., 1994; Cooley et al., 1995), few published reports of gene silencing in transgenic monocots exist. Although an association between the presence of multiple copies of introduced gene sequences and lack of expression has been reported for maize (Register et al., 1994) and rice (Rathore et al., 1993; Cooley et al., 1995), no insight into the mechanism of silencing was obtained, other than the observation that low levels of GUS expression in stably transformed maize calli were correlated with methylation of the coding region (Klein et al., 1990). Similarly, in a recent study a large number of transgenic rice plants produced by biolistics were analyzed and revealed both non-Mendelian inheritance of hygromycin resistance and suppression of

GUS expression (Qu et al., 1996), although the molecular basis was not investigated.

Methylation has been implicated as a component of a dynamic developmental regulatory system responsible for the change of state and transcriptional repression of maize transposable elements (Banks et al., 1988; Fedoroff, 1989). It has also been associated with paramutation (a natural example of *trans*-inactivation; Brink, 1973; Martienssen, 1996; Matzke et al., 1996) in maize. Methylation has often been associated with transgene inactivation in dicots (Finnegan and McElroy, 1994), and there is mounting evidence that de novo methylation is involved in gene silencing (Flavell, 1994).

In the present study genomic analysis of R_1 progeny from the bialaphos-resistant transgenic rice line JKA 52 revealed that, despite the presence of at least two intact copies of *bar*, together with numerous fragmented or rearranged copies, several were herbicide-sensitive. This indicated that expression was silenced in these progeny, but rearrangement of *bar* was unlikely to be the cause, since analysis with several restriction enzymes yielded identical hybridization patterns for both sensitive (silenced) and resistant plants (Figs. 5 and 6 and data not shown). However, substantial methylation of the *Ubi1* promoter and *bar*-coding region was evident for all of the silenced plants (Fig. 7).

Whereas none of the herbicide-sensitive plants contained unmethylated promoter fragments (Fig. 7A), at least one unmethylated copy of the coding region was present (Fig. 7B), suggesting that promoter methylation was the primary determinant of *bar* inactivation. The 5′ untranslated region of *Ubi1* was also methylated in silenced plants but not in resistant plants (Fig. 7B). Studies on the developmental regulation of the maize *Suppressor-mutator* (Spm) transposable element revealed that its phase setting is determined by the methylation of sequences 5′ of the transcription start site, whereas the developmental program is determined by the methylation pattern within the 80% GC-rich sequence of its first untranslated exon (Banks et al., 1988). However, to our knowledge, methylation of the 5′ untranslated region in a silenced transgene locus has not been reported previously in plants.

cis-Inactivation and Transcriptional Silencing

Although multiple copies of the *bar* transgene are present in JKA 52, they appear to be present at a single locus, based on segregation and genomic analysis of herbicide-resistant progeny and the absence of vector or transgene-specific sequences in recessive progeny. This is consistent with previous findings in transgenic animals and plants that concatenation and rearrangement of foreign DNA occur prior to integration in the genome (Gordon-Kamm et al., 1990; Spencer et al., 1990; Dorer, 1997). Several instances of cis-inactivation resulting from the presence of multiple copies of homologous sequences (repeat induced gene silencing) have been reported for plants (Mittelsten Scheid et al., 1991; Kilby et al., 1992; Assaad et al., 1993), and the presence of multiple partial and rearranged copies of *Ubi1/bar* in JKA 52 (Fig. 4B) make it likely that the observed

silencing results from a homology-mediated mechanism. However, since the transgene locus in JKA 52 is relatively complex, the possibility that the silencing of *bar* is also due to heterochromatinization of transgene arrays (because of the presence of repeated sequences or physical proximity of the transgene locus to endogenous heterochromatic regions), similar to the findings in *Drosophila* (Sabl and Henikoff, 1996; Dorer, 1997), was not ruled out. The fact that all of the silenced lines were homozygous further suggests the involvement of homology-based silencing, since pairing of alleles in the homozygote could have further enhanced inactivation of the complex JKA 52 locus already potentiated for silencing.

Homology-mediated gene silencing based on DNA-DNA pairing (Rossignol and Faugeron, 1994) has been postulated to operate via direct or indirect DNA-DNA interactions (Matzke and Matzke, 1995). A major distinction between these two processes is that, whereas direct interactions are thought to require at least 300 bp of homology (Matzke and Matzke, 1995), as little as 90 bp are adequate to stimulate silencing by indirect interactions (Vaucheret, 1993). Since the data shown in Figure 4B reveal the presence of two fragments of at least 600 bp and several smaller fragments with homology to the intact promoter, either process may have triggered methylation and silencing in the progeny from JKA 52. No sequence for rice corresponding to maize *Ubi1* (used to drive the *bar* gene) has been published. Any promoter homology must be low, since several genomic blots in which the maize *Ubi1* promoter was used as a probe did not show any hybridization to wild-type rice genomic DNA (e.g. see lane wt in Fig. 7A). This makes it unlikely that the native rice *Ubi1* (if present) has sufficient homology to the maize *Ubi1* promoter to be affected by silencing.

Transcriptional inactivation and posttranscriptional processes are both known to be involved in gene silencing (Matzke and Matzke, 1995). RNase protection assays revealed the presence of *bar* mRNA in bialaphos-resistant parental (JKA 52) and progeny (52–6) plants, but no *bar* mRNA was detected in the silenced plants. Nuclear run-on transcription experiments confirmed that the lack of *bar* transcripts in silenced lines was due to transcriptional inactivation (Fig. 8B). These experiments also established that antisense effects from any rearranged inserts are unlikely because of transcriptional silencing at the insertion locus. These data confirm and extend recent findings in tobacco (Park et al., 1996), in which transcriptional silencing was found to be associated with increased promoter methylation. Taken together, these findings support the concept that promoter (and 5' untranslated region) methylation is induced by the presence of multiple gene fragments and can provoke transcriptional inactivation.

As mentioned in "Results," hybridization of genomic DNA from the transgenic plants with a vector sequence probe yielded a smear of hybridization, commensurate with the integration of many fragmented and rearranged vector sequences. The hybridization to vector sequences shown in Figure 8B for JKA 52 and JKA 52–6 probably reflects the transcription of vector sequences that fortuitously lie downstream of *Ubi1* or 35S promoter elements. The lack of hybridization to vector sequences seen in the silenced progeny (JKA 52–9 and JKA 52–10) indicates silencing of the entire transgene locus, and analysis of the organization and methylation status of this region is under investigation.

Heritability and AzaC-Mediated Reactivation of the Methylated State

Silencing of the *bar* gene was first observed in the R_1 seedlings derived from selfing the bialaphos-resistant R_0 plant, leading to the question of when the methylation of the *bar* gene occurred. Comparison of the hybridization patterns for *Hpa*II and *Msp*I digests probed for promoter and coding regions (Fig. 7, A and B, respectively), revealed that several sites were methylated in the primary transformant and that at least some methylation occurred in somatic cells. However, the degree of methylation is greater in the resistant progeny (52–6) and even more so in the sensitive progeny (52–9, 52–10, and 52–15), suggesting that extensive de novo methylation occurred during reproduction.

Studies of *Neurospora crassa* and *Ascobolus inmersus* have revealed two types of premeiotic, repeat-mediated gene inactivation: repeat-induced point mutation and methylation induced premeiotically (Rossignol and Faugeron, 1994). It has been proposed that gene silencing in plants, if mediated by an analogous homology-based mechanism, should be active in somatic cells (Flavell, 1994), and our data are consistent with this proposition but also suggest that further methylation and silencing occur during reproduction or possibly during the late vegetative phase.

All R_2 progeny derived from the silenced R_1 plants were sensitive to bialaphos, indicating meiotic transmission of the inactivated state. This is in agreement with studies in dicots (Matzke et al., 1994a), in which silenced genes were inherited and maintained in the inactive state through successive generations. In the presence of AzaC, however, herbicide resistance was restored in several seedlings, indicating reactivation of *bar*. Molecular analyses (Fig. 7A) revealed that at least one demethylated, functional copy of the *Ubi1* promoter was available in the reactivated line 52–10–16 to drive *bar* gene expression, corroborating a functional relationship between demethylation and transcriptional reactivation, as suggested previously (Kilby et al., 1992; Renckens et al., 1992). In the nonreactivated line 52–10–8 very faint bands were visible at the expected locations. Since the methylation observed in the present study was extensive and previous studies of dicots revealed that AzaC-mediated demethylation is random and never complete (Kilby et al., 1992; Renckens et al., 1992), we believe that most or all of the critical cytosine sites are still methylated in line 52–10–8 and contributed to its herbicide sensitivity.

Biotechnological Implications of Homology-Mediated Gene Silencing

Gene silencing is a concern for biotechnological applications, in which reliable expression of an introduced gene is essential for maintenance of the desired trait(s) (Meyer, 1995). The present demonstration of gene silencing in a monocot extends previous observations for several dicots

and suggests that this phenomenon is ubiquitous in plants. To date, examples of methylation being implicated in gene regulation in monocots are restricted to endogenous sequences, i.e. regulation of transposons (Banks et al., 1988; Fedoroff, 1989) and paramutation in maize (Brink, 1973; Martienssen, 1996; Matzke et al., 1996). By providing data concerning methylation of the introduced sequences, our study not only adds transgenes to this category but also suggests the existence of common mechanisms for instigating methylation.

The evidence for partial methylation of DNA in herbicide-resistant plants (Fig. 7) suggests that the inserted locus is potentiated for silencing. Furthermore, aberrant ratios found here (e.g. 1:1 for R_1 progeny of JKA 52) imply that the mechanism by which gene inactivation is fully triggered may be complex and possibly affected by external factors such as environmental conditions. In agreement with the concept that the presence of multiple homologous sequences targets the transgenic locus for silencing, progeny of rice lines containing a single copy (Battraw and Hall, 1992) or relatively few copies (JKA 58, JKA 60; Fig. 4) of the inserted gene have shown no evidence for silencing.

Most cases of gene silencing appear to result from the presence of multiple copies of a transgene. Unfortunately, the integration of multiple rearranged copies of input DNA is a characteristic common to all direct DNA uptake-mediated transformation systems. In contrast to biologically directed transformation, such as that by A. tumefaciens, direct DNA uptake usually also results in the introduction of plasmid vector sequences. We observed a much higher frequency of vector fragment integration compared with promoter- or coding-region integration in our transgenic rice. It seems likely that vector fragments also contributed to gene silencing because of the additional regions of homology (Matzke et al., 1994a). Alternatively, since the plasmid vector and bar gene DNA are of prokaryotic origin, they could have been recognized and inactivated (methylated) by a mechanism that evolved to protect higher eukaryotes from invading pathogens (Bestor, 1990; Doerfler, 1991; Clark et al., 1997).

Although seminal work on cosuppression in plants involved transformation with plant genes under the control of their own promoter (van der Krol et al., 1990) or the 35S promoter (Napoli et al., 1990; van der Krol et al., 1990), most studies of plant gene silencing involve viral (35S) or bacterial (e.g. Ti plasmid-derived nos) promoters (Vaucheret, 1993; Matzke et al., 1994a). The present observations show silencing in rice obtained in the presence of the ubiquitin-1 (Ubi1) promoter, a strong promoter from another monocot plant. They extend the relatively few reports of silencing in dicots that involve plant promoters other than 35S and strengthen the notion that silencing results from a mechanism or mechanisms that are generally present in higher plants.

Our findings strongly support the concept that gene silencing can result from the introduction of multiple homologous sequences (Matzke et al., 1996) and emphasize the need for the careful assessment of the organization and methylation status of the transgene loci. Al-

though not documented in the detail provided here, we have observed a high frequency of silencing for several different transgenes in rice. Additionally, in other experiments we have observed rearrangement and deletion of fragments of transgene inserts. Thus, it is not unreasonable to speculate that organisms with a small genome (like that of rice) contain very active systems for sensing intrusive DNA and that tagging and inactivation by methylation may represent early processes in the elimination of alien genetic information.

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