Transposon-Mediated Single-Copy Gene Delivery Leads to Increased Transgene Expression Stability in Barley[1]

Thomas Koprek[2], Sergio Rangel, David McElroy[3], Jeanine D. Louwerse[4], Rosalind E. Williams-Carrier[5], and Peggy G. Lemaux*

Department of Plant and Microbial Biology, University of California, Berkeley, California 94720

Instability of transgene expression in plants is often associated with complex multicopy patterns of transgene integration at the same locus, as well as position effects due to random integration. Based on maize transposable elements *Activator* (*Ac*) and *Dissociation* (*Ds*), we developed a method to generate large numbers of transgenic barley (*Hordeum vulgare* var Golden Promise) plants, each carrying a single transgene copy at different locations. Plants expressing *Ac* transposase (*Ac*TPase) were crossed with plants containing one or more copies of *bar*, a selectable herbicide (Basta) resistance gene, located between inverted-repeat *Ds* ends (*Ds-bar*). F_1 plants were self-pollinated and the F_2 generation was analyzed to identify plants segregating for transposed *Ds-bar* elements. Of *Ds-bar* transpositions, 25% were in unlinked sites that segregated from vector sequences, other *Ds-bar* copies, and the *Ac*TPase gene, resulting in numerous single-copy *Ds-bar* plants carrying the transgene at different locations. Transgene expression in F_2 plants with transposed *Ds-bar* was 100% stable, whereas only 23% of F_2 plants carrying *Ds-bar* at the original site expressed the transgene product stably. In F_3 and F_4 populations, transgene expression in 81.5% of plants from progeny of F_2 plants with single-copy, transposed *Ds-bar* remained completely stable. Analysis of the integration site in single-copy plants showed that transposed *Ds-bar* inserted into single- or low-copy regions of the genome, whereas silenced *Ds-bar* elements at their original location were inserted into redundant or highly repetitive genomic regions. Methylation of the non-transposed transgene and its promoter, as well as a higher condensation of the chromatin around the original integration site, was associated with plants exhibiting transgene silencing.

The successful introduction of transgenic crops into modern farming practices depends on maintaining and improving the agricultural performance of the modified plants and on the stable and predictable transmission and expression of the transgene in successive generations during seed production and commercial cultivation (McElroy, 1999). Inactivation of transgene expression has often been observed in plants and seems to be especially problematic in cereal crops. In these crop species the most frequently used biolistic transformation methods typically result in complex, multicopy transgene integration patterns, where it has been observed that more than 50% of T_1 plants exhibit transgene silencing (Wan and Lemaux, 1994; Pawlowski et al., 1998; for review, see Iyer et al., 2000). Inactivation of expression in plants with single copies of the transgene can occur (Meyer and Heidmann, 1994), but it is not as frequent as the silencing observed when multiple copies of the transgene exist.

In addition to effects of copy number on transgene silencing (Assaad et al., 1993; Atkinson et al., 1998), this phenomenon is influenced by many other factors. These include the presence of inverted repeats in the complex integration patterns (Stam et al., 1997a, 1997b), the overexpression of the transgene (Que et al., 1997), the nature of the insertion site (Matzke and Matzke, 1998), the AT/CG composition of the transgene (Matzke and Matzke, 1998), and environmental factors (Meyer et al., 1992). These factors can trigger different mechanisms of transcriptional or post-transcriptional gene silencing.

Due to the exacerbated problems associated with transgene silencing, and especially silencing in multicopy insertion events, it is desirable to generate transgenic plants containing only a single copy of the transgene. With existing cereal transformation methods, however, the number of single-copy transgenic plants generated is usually low relative to the number of plants containing multicopy events. This is particularly problematic in crop species that are not readily transformed, since obtaining the large numbers of independent transformants to find the infrequent single insert is time consuming and costly. Furthermore, although it is possible with sufficient

[2] Present address: Max Planck Institut für Züchtungsforschung, Carl-von-Linne-Weg 10, 50829 Köln, Federal Republic of Germany.

[3] Present address: Maxygen, Inc., 515 Galveston Drive, Redwood City, CA 94063.

[4] Present address: International Centre for Brewing and Distilling, Herriot-Watt University, Riccarton, Edinburgh EH14 4AS, Scotland, UK.

[5] Present address: Institute of Molecular Biology, University of Oregon, 270 Onyx Ridge, Eugene, OR 97403.

* Corresponding author; e-mail lemauxpg@nature.berkeley.edu; fax 510–642–7356.

effort to identify a sufficient number of single-copy insertion events, this approach alone will not always overcome transgene expression instability problems. Transgene silencing has also been observed in events with single, simple-pattern transgene inserts associated with, for example, location of the insert, GC content of the region, and presence of vector DNA.

Attempts have been made to increase the number of single-copy transgenic plants. In general, *Agrobacterium*-mediated transformation leads to lower copy numbers of the transgene, and single-copy inserts in transgenic plants are observed more frequently. For some cereals, however, this method is not routine. Even though single-copy plants can be obtained more frequently in dicot and monocot (Cheng et al., 1997; Tingay et al., 1997) species, the use of *Agrobacterium*-mediated transformation does not circumvent the problem of transgene silencing (e.g. Ishida et al., 1996; Park et al., 1996). Other methods have been employed to increase the number of single-copy inserts, including the use of an Agrolistic method (Hansen and Chilton, 1996) and inhibitors of recombination (De Block et al., 1997). Another successful approach has been used in wheat and utilizes the bacteriophage *cre/lox* recombination system to resolve the complex integration patterns of multiple transgenes into single-copy integration patterns (Srivastava et al., 1999). However, these approaches do not always overcome transgene expression stability problems.

We developed a system in barley (*Hordeum vulgare* var Golden Promise) based on the maize transposons *Activator* (*Ac*) and *Dissociation* (*Ds*). In this system the transgene is inserted between the nonautonomous *Ds*-element inverted repeats and is translocated to different loci in the genome as a result of the action of *Ac* transposase (*Ac*TPase). The *Ds* cassette carries *bar* (*Ds-bar*), which encodes phosphinothricin acetyl transferase, which confers resistance to the herbicide, Basta. This cassette can transpose to genetically unlinked sites, which segregate in the next generation from the remaining transgene copies, the other vector sequences, and the *AcTPase* gene. This method requires only a few original transformation events and results in high numbers of single-copy transgenic plants, each carrying a stabilized transgene at different genomic locations. Transposons are also subject to silencing (Fedoroff and Chandler, 1994), although at much reduced frequencies relative to most transgenes. Here we discuss the influence of transgene copy number and the nature of the integration site of the transposed *Ds-bar* element on transgene expression stability.

RESULTS

Single-Copy Transgenic Plants

From crosses between plants expressing functional *Ac*TPase and plants from three independent transfor-

mants that contain one to six copies of *Ds-bar* we obtained numerous families of F_1 plants carrying both elements. Progeny of four self-pollinated F_1 plants were analyzed (200 F_2 plants derived from line A8-1 and 100 F_2 plants from A8-5, A1-5, and A18-5) to identify F_2 plants containing a single copy of transposed *Ds-bar* that had segregated away from the gene encoding *Ac*TPase (*AcTPase*) and other copies of *Ds-bar*. These plants were designated "TNP" (transposed) in contrast to segregating plants designated "nTNP" (non-transposed) carrying *Ds-bar* at the original integration sites and also not containing *AcTPase*.

Among 200 F_2 progeny (designated a "family") from F_1 plant A8-1, which contained a single *Ds-bar* element at its original integration site after microparticle bombardment, we identified 21 plants (designated "lines") with a single *Ds-bar* element transposed to a new position, as confirmed by PCR and DNA-blot hybridization analysis. From F_1 plant A8-5, a sibling of A8-1 also carrying a single *Ds-bar* element at its original integration site, we obtained 17 F_2 TNP plants (hereafter referred to as lines). Among progeny of F_1 plants A1-5 and A18-5, which have different and independently transformed parents from the A8-1 and A8-5 families and which contain six and five copies of *Ds-bar*, respectively, we identified in the F_2 generation five TNP lines from each family. Figure 1 shows a DNA-blot hybridization of samples from different F_2 lines derived from A18-5. In this blot the hybridization pattern of DNA from three plants containing a single copy of *Ds-bar* is seen as different-sized *Hin*dIII fragments. Each of the *Hin*dIII sites is unique and is predicted to be in the genomic DNA flanking the site of *Ds-bar* integration. The pres-
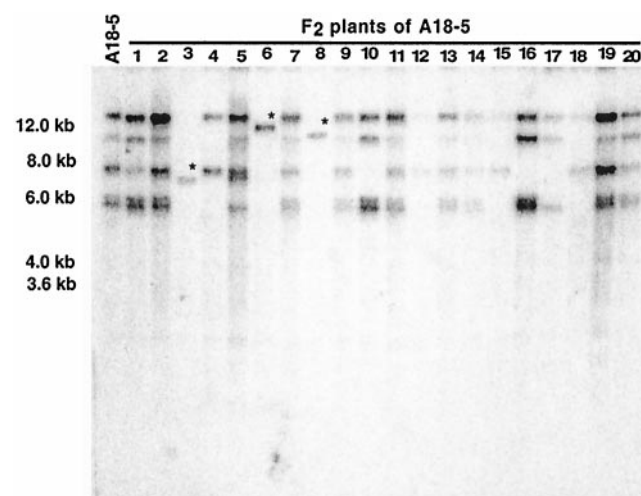

Figure 1. Transposon-mediated generation of single-copy transgenic plants. DNA-blot hybridization shows the integration pattern of *Ds-bar* after *Hin*dIII digest of genomic DNA from F_1 parent, A18-5 (far left lane), and 20 of its F_2 progeny. In three plants (i.e. nos. 3, 6, and 8), a single *Ds-bar* cassette has transposed to an unlinked site, segregating away from other copies. The asterisk above the band marks the position in single-copy *Ds-bar* plants that differs from the parental bands. M_r markers in kilobases are indicated on the left.

ence or absence of *AcTPase* was, in all cases, analyzed by PCR using *AcTPase*-specific primers (data not shown). Other single-copy events were analyzed in an identical manner (data not shown).

Transgene and Transgene Expression Stability

The physical transmission of *bar* from the single-copy TNP lines followed Mendelian segregation in the F_3 generation in the newly created single-copy, *Ds-bar* plants, as evidenced by DNA-blot hybridization analysis of genomic DNA. In the TNP and nTNP lines from families A8-1, A8-5, A1-5, and A18-5, functional expression of the *bar* gene product was analyzed in later generations by monitoring herbicide resistance. While screening the F_2 populations from the four families to identify single-copy *Ds-bar* individuals, plants were painted with Basta. All TNP lines in the F_2 generation were completely resistant to the herbicide. In contrast, up to 40% of all nTNP lines derived from the four families showed reduced numbers of Basta-resistant plants, consistent with transgene silencing.

Transgene-expression stability of lines derived from the four families was monitored in advanced generations in 17 nTNP lines and 35 TNP lines, all of which were Basta-resistant in the F_2 generation. Between 40 and 50 F_3 and F_4 plants, derived from each of the 52 F_2 lines, were tested for herbicide sensitivity. To avoid damage or death of the entire plant in the case of herbicide sensitivity and to ensure seed set, only one leaf of each plant was painted with Basta. We observed no expression of the *bar* gene product in F_3 progeny of 14 of the 17 nTNP lines (82.4%), as evidenced by sensitivity of the plants in the line to Basta (Table I). In the F_3 generation 100% of all nTNP lines derived from multicopy families A1-5 and A18-5 contained plants in which Basta sensitivity was evident. In contrast, only six of the 35 TNP lines (17.1%) produced F_3 progeny showing transgene silencing (Table I). The number of plants within nTNP lines that showed evidence of transgene silencing was significantly higher statistically ($P <$ 0.1%; chi-square analysis) than within TNP lines (Table I). Even though only seeds from Basta-resistant F_3 plants were collected, transgene silencing continued to persist into the F_4 generation. Only one of 17 nTNP lines produced completely resistant F_4 plants; 94% of

all nTNP lines contained between 1% and 48% of plants with silencing of the *bar* gene product (Table I; Fig. 2). It is striking that all 29 TNP lines, which were completely Basta-resistant in F_3, also produced completely resistant F_4 plants. Among F_4 progeny from the six TNP lines that exhibited transgene silencing in the F_3 generation, plants were again Basta-sensitive in numbers comparable with those in the nTNP lines (data not shown). Progeny of one TNP plant line (A1-5-54) derived from family A1-5 were completely Basta-resistant in the F_3 generation, but showed signs of transgene expression instability in F_4.

Analysis of the Integration Sites of *Ds-bar*

To analyze the redundancy of the genomic region into which the *Ds-bar* element from single-copy nTNP and TNP plants had inserted, DNA blots of *Hin*dIII-digested DNA from non-transgenic barley plants (var Golden Promise) were hybridized with probes from genomic regions flanking the *Ds* element. Flanking regions were isolated from the single-copy nTNP plant (A8-1-5), three silenced TNP plants (e.g. see Fig. 3, A8-1-51), and six stably expressing TNP plants (e.g. see Fig. 3, A8-1-22, -34, -85, and -120) that have *Ds-bar* in different locations. Analysis of the DNA blots showed that the single-copy *Ds-bar* element from the silenced nTNP plant A8-1-5 had integrated into a highly repetitive genomic region. Two of the three TNP plants with silenced transgene expression (e.g. see Fig. 3, A8-1-51) had transposed into highly or moderately repetitive regions of the barley genome. The *Ds-bar* elements from all stably expressing TNP plants had transposed to single-copy or low-copy regions (up to three hybridizing fragments) of the barley genome (e.g. Fig. 3, A8-1-22, -34, -85, and -120 and data not shown). Only one line, A8-1-25, in which *Ds-bar* had inserted into a non-repetitive genomic region (data not shown), exhibited signs of transgene silencing and the silencing was observed in only one of 31 (3%) *bar*-containing F_4 plants.

Methylation Analysis of the Promoter and Coding Region of *Ds-bar*

To assess the methylation status of the *Ds-bar* element a series of DNA hybridization blots were con-

Table I. *Transgene expression stability in nTNP and TNP plant lines*

F_1 Plant	No. of Copies	No. of Lines with Transgene Expression Instability/Total Lines Analyzed					
		F_2		F_3		F_4	
		nTNP[a]	TNP[b]	nTNP	TNP	nTNP	TNP
A8-1	1	6/8 (75%)	0/15 (0%)	6/8 (75%)	2/15 (13%)	7/8 (88%)	2/15 (13%)
A8-5	1	4/5 (80%)	0/10 (0%)	4/5 (90%)	2/10 (20%)	5/5 (100%)	2/10 (20%)
A1-5	6	N.D.	N.D.	2/2 (100%)	1/5 (20%)	2/2 (100%)	2/5 (40%)
A18-5	5	N.D.	N.D.	2/2 (100%)	1/5 (20%)	2/2 (100%)	1/5 (20%)

[a] nTNP lines are "non-transposed," containing *Ds-bar* at the original integration site and do not contain A*c*TPase. [b] TNP lines are "transposed" and are progeny of F_1 plants selected for a single copy of *Ds-bar* transposed to a new location.
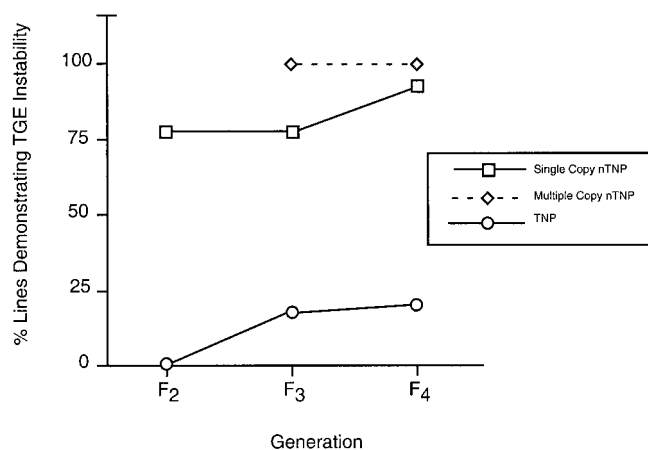
Figure 2. Average frequency of transgene expression instability in F_2, F_3, and F_4 populations. Lines from F_2, F_3, and F_4 populations were assessed for functional expression of the product of *bar* by testing for Basta sensitivity. Percentages represent the number of lines in which at least one plant exhibited Basta sensitivity divided by the total number of lines per family tested. TNP, Transposed *Ds-bar* element, no *ActPase*; nTNP, *Ds-bar* element remaining at original site of integration following bombardment, no *AcTPase*; TGE, transgene expression.

structed to analyze the *ubiquitin* promoter region driving *bar* gene expression and the *bar*-coding region itself. DNAs from different silenced and non-silenced nTNP and TNP plants were digested with six different methylation-sensitive enzymes, and hybridization patterns with multiple probes were analyzed to provide information about the methylation status at specific restriction sites. The data in Figure 4A shows the results of digestion with *Hpa*II; the positions of restriction sites located within the *ubiquitin* promoter and first intron region are indicated in Figure 4B. All DNA samples from Basta-sensitive plants showed evidence of methylation at certain *Hpa*II sites in the *ubiquitin* promoter region. In contrast, DNA samples from Basta-resistant plants showed no evidence of methylation at these *Hpa*II sites. Based on the map position of *Hpa*II sites (Fig. 4B) and predicted fragment sizes after *Hpa*II digestion, it appears that certain sites are more prone to becoming methylated than are others. For example, *Hpa*II sites I and III were non-methylated in all plants shown in Figure 4A, resulting in fragment sizes of 850 or 1,150 bp after hybridization with probe A (Fig. 4B). Analysis of additional plants, however, showed that in a few cases *Hpa*II sites I and III were hyper-methylated (data not shown). In contrast to that situation, *Hpa*II sites II and V were always methylated in silenced plants, as confirmed by hybridization with probes B and C (data not shown).

Restriction digests with other methylation-sensitive enzymes showed an increased level of methylation in the promoter region in all silenced plants (data not shown). The methylation pattern within the analyzed promoter/intron region varied

from plant to plant, but, in general, was higher in Basta-sensitive F_4 plants than in F_3 plants. Figure 4B shows a diagram of the analyzed promoter/intron region with the sites indicated that were found to be methylated in at least some plants. In most silenced plants only a few of the sites shown in Figure 4B were methylated or partially methylated; however, in non-silenced plants none of these sites were methylated. The highest degree of methylation was found in silenced single-copy nTNP plants and in silenced TNP plants in which the transposed *Ds-bar* element had reinserted into highly repetitive genomic regions (e.g. Fig. 3, plant A8-1-51).

DNase I Sensitivity Tests

To determine if the silencing observed in nTNP and TNP plants is correlated with changes in the contiguous chromatin structure, the DNase I sensitivity of the transgene region was determined using isolated nuclei from several silenced and non-silenced plants.
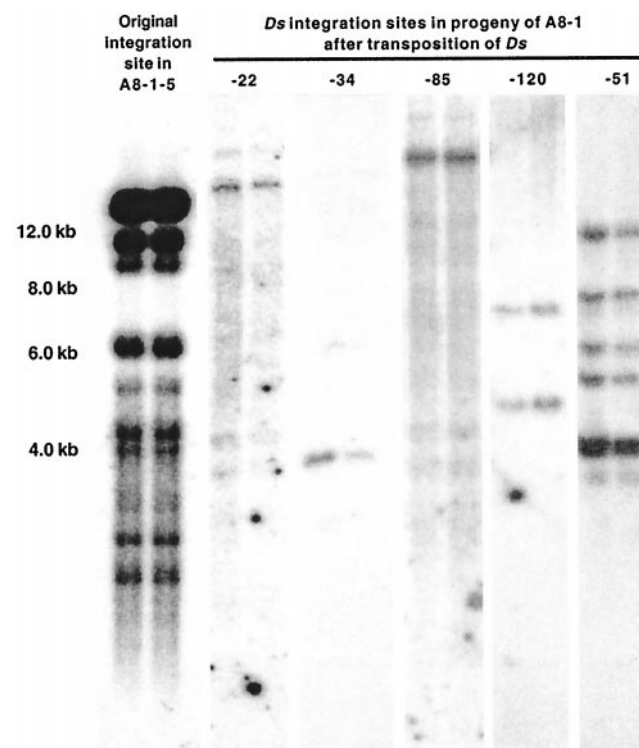

Figure 3. Analysis of *Ds-bar* integration sites. Genomic DNA flanking single-copy *Ds-bar* elements was isolated from six different transgenic plants by thermal asymmetric interlaced-PCR. PCR fragments were then used as hybridization probes with DNA blots of *Hind*III-digested genomic DNA from non-transgenic plants. The two lanes on left are results after hybridization with a probe specific to the genomic DNA flanking the original *Ds-bar* insertion site of line A8-1-5. Other sets of lanes show results after hybridization with probes flanking the insertion sites from five lines in which *Ds-bar* has transposed to a new location: four lines that stably express the transgene, e.g. nos. 22, 34, 85, 120, and one line that does not, 51. M_r markers in kilobases are indicated on left.
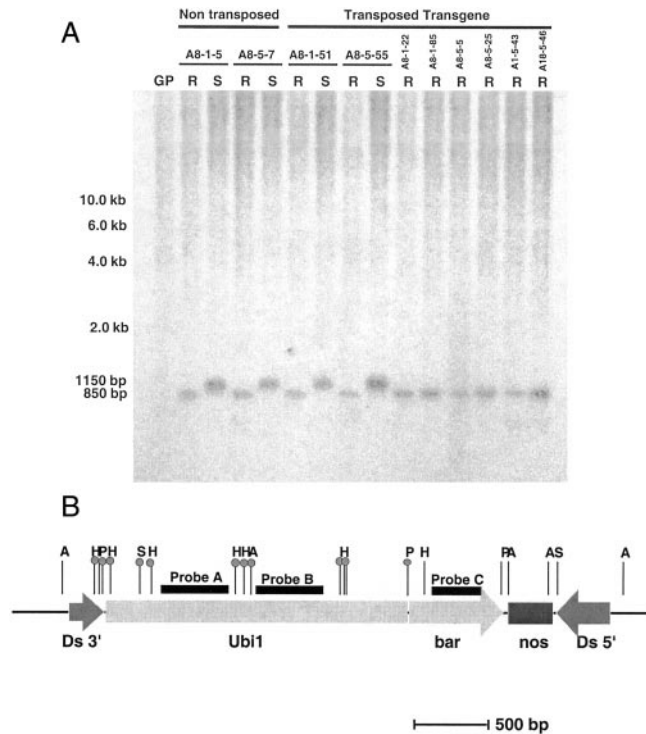
Figure 4. A, Methylation analysis of Basta-sensitive and Basta-resistant plants from lines with transposed and non-transposed *Ds-bar* cassette. DNA blots of *Hpa*II-digested genomic DNA from Basta-sensitive and Basta-resistant plants were hybridized with probe A (B) from the 5′ end of the ubiquitin promoter of the *Ds-bar* cassette. Lanes containing DNA from Basta-resistant plants have an 850-bp hybridization fragment, as expected for a non-methylated *Hpa*II site in the *ubiquitin* promoter region. Digests of DNA from Basta-sensitive plants have fragments of 1,150 bp, indicating methylation of an *Hpa*II site in the immediate 5′ region of probe A (B). R, Basta-resistant; S, Basta-sensitive; GP, Golden Promise non-transgenic control. B, Methylation analysis of promoter and coding region of *Ds-bar* cassette in Basta-sensitive lines. Diagram summarizes results of digests with different methylation-sensitive enzymes (A, *Ava*I; H, *Hpa*II/*Msp*I; P, *Pst*I; Sc, *Sac*II; and S1, *Sal*I) and hybridization with three different probes (A, B, and C). *Hpa*II/*Msp*I sites are numbered I through VII from left to right. A total of 20 different Basta-sensitive plants from eight different lines were analyzed. Based on the size of hybridization fragments and information regarding the restriction sites in the *Ds-bar* cassette, the position of specific methylated sites was determined. In some cases different *Hpa*II/*Msp*I sites are not sufficiently separated to allow differentiation between individual sites. In accordance with this, closely connected sites were marked as methylated if at least one of the sites was methylated. A circle on top of the lines symbolizes methylation was detected at that site in at least some plants. Size bar indicates 500 bp.

Chromatin of two silenced nTNP plants, four silenced TNP plants, and five non-silenced plants was analyzed. Figure 5 shows the results of a representative experiment. The hybridization signal of the expected 1.9-kb fragment in nTNP plant A8-1-5, which shows unstable expression and has a single *Ds-bar* element, decreases slowly over a 20-min period, indicative of low accessibility of the transgene region to DNase I. In contrast, in TNP line A8-1-85, which

exhibits stable expression of the transgene, the hybridization signal decreases rapidly and disappears completely after a 15-min incubation period with DNase I. The transgene of a silenced TNP plant (A8-1-51) exhibited a similar low accessibility to DNase I as that observed in silenced nTNP plants. Analysis of the comparison of the DNase I sensitivity of different silenced and non-silenced TNP plants showed that silencing was associated with a compact chromatin structure in the vicinity of the transgene in 10 of 11 analyzed plants. The chromatin of one silenced TNP plant showed a DNase I sensitivity that was comparable with the sensitivity of chromatin in non-silenced TNP plants. The accessibility to DNase I varied between different non-silenced TNP lines, but was very similar between individual plants from the same TNP line (data not shown).

DISCUSSION

Transgene expression instability is a common phenomenon in genetically transformed plants from monocot species (Iyer et al., 2000). The majority of cases of transgene silencing can be correlated with the existence of multicopy inserts, sequence rearrangements, and the random nature of integration into the genome, all of which occur frequently when methods of direct gene transfer are used for transfor-
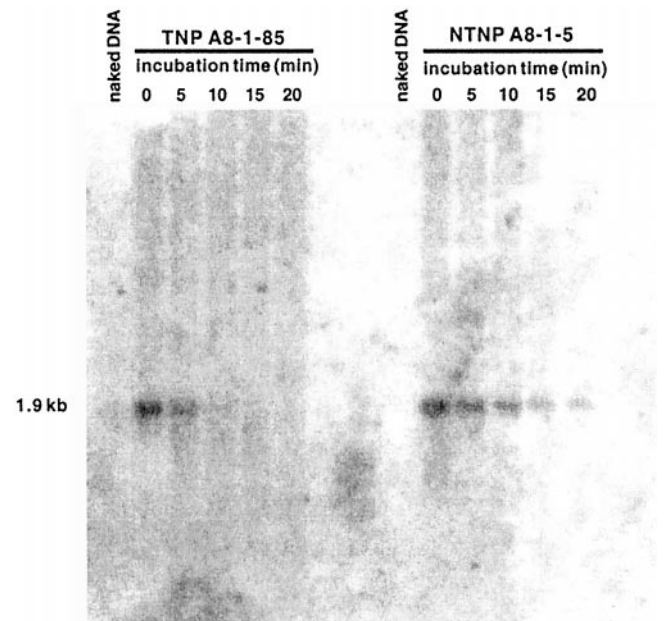

Figure 5. Analysis of chromatin structure near *Ds*-bar cassettes from lines with a transposed or non-transposed *Ds*-bar cassette. Intact nuclei from plants with (i.e. TNP A8-1-85) or without (i.e. nTNP A8-1-5) transposed *Ds*-bar were treated with DNaseI for 0 to 20 min. DNA was isolated, digested with *Bgl*I to obtain fragments of a specific reference size (1.9 kb), electrophoretically separated, blotted, and hybridized with probe C (Fig. 4B). Naked DNA (no intact chromatin) from both plants, treated for 2 min with DNase I, served as a control.

mation (Hiei et al., 1994). Transgene silencing occurs in single-copy transgenic plants due to other factors such as insertion position, but the silencing is much less frequent than in multicopy plants. With most present methods of transformation, it is often necessary to screen large numbers of independent transformants to identify single-copy transgenic plants. This makes the process labor intensive, expensive, and, in recalcitrant species, essentially not feasible. These facts make the development of a single-copy gene delivery system that inserts genes into transcriptionally active regions of potential value.

Agrobacterium-mediated gene delivery in cereals leads to lower copy-number insertions, and single-copy transgenic plants are more frequent (Cheng et al., 1997; Tingay et al., 1997). This method, however, does not eliminate the problem of gene silencing in cereals (Ishida et al., 1996; Park et al., 1996) because even *Agrobacterium*-generated transgenic plants containing a single-copy of the transgene showed evidence of silencing (Waterhouse et al., 1998).

In the present study we describe a different method of generating large numbers of single-copy transgenic plants in barley by crossing *Ac*TPase-expressing plants with plants containing one or multiple *Ds*-elements into which an herbicide resistance gene was inserted. We show that a single *Ds-bar* element can transpose from a multicopy locus to linked and unlinked sites. In the latter case the element can segregate away from the other copies of the *Ds* element and from the *Ac*TPase gene (Koprek et al., 2000), physically stabilizing the element in its new location. The resulting single-copy element can be re-activated by providing *Ac*TPase in *trans.* In this way, hundreds of independent, single-copy transgenic plants can readily be generated and the transgene of interest can be separated away from the rest of the vector sequences, e.g. plasmid backbone and selectable marker genes.

Analysis of transgene expression in plants carrying *bar* in new locations following transposition showed that plants from most TNP lines were completely Basta-resistant in the F_3 and F_4 generation, exhibiting no signs of necrosis after herbicide treatment. In contrast, a large proportion of lines with one or multiple copies in their original integration site (82% in F_3 and 94% in F_4) showed signs of transgene inactivation. This striking difference occurred in the F_4 generation despite the fact that only Basta-resistant F_3 plants were chosen for generation-advance. In all cases studied, gene-silenced plants exhibited increased methylation of cytosine residues in the *ubiquitin* promoter region linked to *bar* and a more condensed chromatin structure in the immediate vicinity of the transgene or the transposed silenced *Ds*-bar element. These observations are consistent with the observations of others that transcriptional gene silencing often correlates with changes in the methylation status (Stam et al., 1997a, 1997b) and with a locally condensed chromatin structure (Ye and Signer, 1996; van Blokland et al., 1997).

Despite the dramatic improvement in transgene expression stability in the TNP plants, silencing was observed in a few TNP lines in our experiments, indicating that additional factors play a role in transgene expression stability. The *Ds-bar* construct contains short, inverted repeats and inverted repeats are known to induce transcriptional and posttranscriptional gene silencing (for review, see Muskens et al., 2000). In addition, methylation-induced, heritable, and reversible transposon inactivation has been described for all major maize transposons (Fedoroff and Chandler, 1994). This phenomenon can be triggered by the terminal inverted repeats of the transposon, as well as by the transposon copy number. Although copy number is not a factor, the inverted repeats are present on the cassette. This mechanism could account for some of the silencing in TNP and nTNP lines in our experiments. However, since all TNP plants, whether silenced or not, contain the same terminal inverted repeats and subterminal repeats of *Ds* and in the same structural context in the single intact *Ds* copy, this would not seem to explain the differences in expression stability among plants from the same line. The analysis of transgene expression in advanced generations will show if the *Ds-bar* elements go through cycles of silencing that are typical for maize transposable elements. To date, tracking transgene expression stability through the F_6 generation in numerous lines has turned up little evidence of transgene expression instability (T. Koprek and S. Rangel, unpublished data).

Another factor that might play a decisive role in transgene expression stability is the nature of the chromosomal location of the transgene insertion, which cannot be controlled and varies from line to line. In our experiments most transposed *Ds-bar* elements tested (80%) re-inserted into single-copy genomic DNA regions and all but one of these lines produced stably expressing progeny. The existence of the one unstable single-copy line indicates that apart from insertion into genomic DNA of low redundancy, other factors like vicinity to matrix attachment regions (Spiker and Thompson, 1996; Holmes-Davis and Comai, 1998), isochore compatibility of the transgene with the flanking genomic DNA (Kumpatla et al., 1998; Matzke and Matzke, 1998), and higher-order chromatin structure (Sun and Elgin, 1999) might influence transgene expression, but these other factors do not appear to affect transgene expression stability in many *Ds*-delivered lines.

Analysis of chromosomal DNA from TNP lines A8-1-51 (Fig. 3) and A8-5-55 (data not shown) in which *Ds-bar* reinserted into repetitive DNA sequences showed a high degree of methylation in the *ubiquitin* promoter region of the transgene (Fig. 4, A and B) and plants derived from these lines showed

signs of transgene silencing. Insertion of transgenes in or near repetitive DNA or heterochromatic regions has been shown to correlate with inactivation of the transgene (Pröls and Meyer, 1992). The results of gene-tagging experiments with *Ac/Ds* in Arabidopsis and tomato show that *Ds* elements frequently transpose into transcriptionally active regions (Parinov et al., 1999; Meissner et al., 2000), and this predisposition could be an explanation for the high percentage of TNP plants that express *bar* stably over several generations.

Our results show that the use of transposable elements as a transporter for transgenes leads to the generation of large numbers of independent single-copy transgenic plants from only two original transformants, one with *Ds* and one with transposase—a situation that has positive implications for recalcitrant species and varieties within a species. In addition, the increased stability of transgene expression in most TNP plants, as well as the ability to eliminate selectable marker genes and other vector sequences, gives rise to a system of gene delivery that has great utility for generating crop plants with improved agronomic characteristics. The system described here can also be used as a valuable tool to gain further insight into mechanisms of gene silencing.

MATERIALS AND METHODS

Plasmids

Plasmid pSP-Ds-Ubi-bar containing the *Ds-bar* element consists of the *Streptomyces hygroscopicus* phosphinothricin acetyl transferase gene (*bar*) under control of the *Ubi1* promoter and first intron from maize and the *nos* terminator as a 0.9-kbp *Cla*I-*Not*I restriction fragment. The *UbiI-bar-nos* cassette is flanked by a 254-bp 5′ sequence and a 340-bp 3′ sequence from *Ds*, derived from pDs7 (Wirtz et al., 1997) as a 0.59-kbp *Sal*I-*Bam*HI restriction fragment. The construction of the entire plasmid has been described in detail previously (McElroy et al., 1997; Koprek et al., 2000).

Plant Material

Transgenic barley (*Hordeum vulgare* var Golden Promise) plants containing the *Ds* element with a *ubiquitin-bar* expression cassette as an insert or the *AcTPase* gene under transcriptional control of the *ubiquitin* promoter from maize or the putative *AcTPase* promoter region were generated by particle bombardment of immature embryos (Wan and Lemaux, 1994). *AcTPase*-expressing plants were crossed with plants that contained one, five, or six *Ds-bar* copies. Resulting F_1 plants were self-pollinated; F_2 plants in which *Ds* had transposed to an unlinked site and segregated away from linked sequences were identified by DNA-blot hybridization and PCR analysis (Koprek et al., 2000).

Transgene Expression Test

Plants negative for the *AcTPase* gene and containing single or multiple *Ds-bar* copies in their original integration site (non-transposed, nTNP) or a single transposed *Ds-bar* in a new location (transposed, TNP) were treated with the herbicide Basta to monitor expression of *bar* (Wan and Lemaux, 1994). A total of 17 nTNP lines (eight lines derived from A8-1, five lines from A8-5, and two lines each from A1-5 and A18-5) and 35 TNP lines (15 lines derived from A8-1, 10 lines from A8-5, and five lines each from A1-5 and A18-5) were tested. When plants were in the three-leaf stage, 2.5 cm-long leaf segments were painted with an aqueous solution containing 0.5% (v/v) Basta and 0.1% (v/v) Triton X-100 using a cotton swab. Plants showing any sign of necrosis due to the herbicide were scored as sensitive. In F_3 generation, plants with a transposed *Ds-bar* cassette were expected to segregate the *Ds-bar* element in a 3:1 ratio. Any plants scored as sensitive were analyzed by PCR to confirm the presence or absence of the transgene; sensitive plants containing the transgene were scored as silenced. Resistant plants were self-pollinated and progeny of these plants (F_4 generation) were analyzed in an identical manner.

DNA-Blot Hybridization

Genomic DNA was isolated from leaf tissue (Cone, 1989) when plants were in the four-leaf stage. To identify plants containing a single copy of *Ds-bar*, 10 μg of DNA was digested with *Hin*dIII, which does not cut within the 3.6-kb *Ds-bar* fragment. The digested DNA was separated in 0.8% (w/v) agarose gels, transferred to nylon membranes (Bio-Rad, Hercules, CA), and hybridized with radioactively labeled (Promega, Madison, WI) *Ds*-specific probe. Analysis of the methylation status of *bar* and the mapping of its location in non-silenced and silenced plants was performed. Genomic DNA was digested to completion with *Ava*I, *Hpa*II, *Msp*I, *Pst*I, *Sac*II, and *Sal*I (*Hpa*II was purchased from New Enlgand Biolabs, Beverly, MA], all other enzymes were from Promega) alone or in combination with the non-methylation sensitive enzymes *Dra*I or *Bgl*I. Digested DNA was treated as above. Hybridizations were carried out consecutively with *bar* expression cassette probes A, B, and C (Fig. 4B). Blots were stripped after exposure to x-ray film and were re-probed according to manufacturer's instructions.

PCR Analysis

Genomic DNA (0.5 μg) was subjected to PCR amplification in a thermocycler (model PTC-100, MJ Research, Waltham, MA). PCR reactions (50 μL) contained 1× PCR buffer (Promega), 200 μM of each dNTP, 1.5 mM MgCl$_2$, 1 μM primer, 1% (w/v) dimethyl sulfoxide, and 2.5 units of *Taq* DNA polymerase (Promega). The primer pairs used for the *bar*-coding region were BAR5′: (5′-TGC ACC ATC GTC AAC CAC TA-3′) and BAR3′: (5′-ACA GCG ACC ACG CTC TTG AA-3′) and were used to produce a PCR product of 311 bp. PCR reactions were performed with an initial

denaturation step at 94°C for 2 min followed by 10 cycles of a touch-down program with decreasing annealing temperatures from 65°C to 60°C in increments of −0.5°C per cycle for 30 s, an extension at 72°C for 30 s, a denaturation at 94°C for 45 s, a subsequent 25 amplification cycles for 30 s at 60°C, 30 s at 72°C, and 45 s at 94°C, and a final extension at 72°C for 5 min. PCR products were analyzed by gel electrophoresis in 1.1% (w/v) agarose gels.

Isolation and Analysis of Regions Flanking *Ds* Inserts

The redundancy of the DNA flanking the integration site of transposed and non-transposed *Ds*-elements was analyzed by hybridizing DNA blots of *Hin*dIII-digested genomic DNA from non-transgenic var Golden Promise plants with isolated *Ds*-flanking genomic DNA regions. Genomic DNA flanking *Ds*-element inserts from 10 different transposition events was isolated using thermal asymmetric interlaced-PCR (Liu et al., 1995) with random primers as described previously (Koprek et al., 2000). Specific amplified tertiary PCR products were analyzed on 1.5% (w/v) agarose gels, isolated using the QIAquick gel extraction kit (Qiagen, Valencia, CA), and used as probes for DNA-blot hybridizations as described above.

Nuclei Isolation and DNase I Sensitivity Test

Five grams of young leaves were ground in liquid N_2; the powder was resuspended in 25 mL of Hamilton buffer (10 mM Tris-HCl, pH 7.6, 1.14 M Suc, 5 mM $MgCl_2$, and 5 mM β-mercaptoethanol; Hamilton et al., 1972) and the slurry was gently stirred and filtered through three layers of Miracloth (Calbiochem, La Jolla, CA) in 10 mL of the same buffer. The final pellet was resuspended, washed in 5 mL of DNase I digestion buffer (50 mM Tris-HCl, pH 8.0, 0.3 M Suc, 5 mM $MgCl_2$, 1.5 mM NaCl, 0.1 mM $CaCl_2$, and 5 mM β-mercaptoethanol), and centrifuged at 1,000g for 10 min. The pellet was resuspended in 1,550 μL of DNase I digestion buffer and 50 μL of the nuclei suspension was stained with 4′,6-diamino-phenylindole. Nuclei were counted under a fluorescence microscope. All manipulations were carried out at 4°C.

For DNase I digestion, aliquots containing 5×10^5 nuclei (usually in 100–150 μL) were transferred into separate reaction tubes and DNase I digestion buffer was added to a final volume of 200 μL per reaction. Nuclei were treated with 1 unit/mL DNase I (Promega) at 25°C for 0, 5, 10, 15, and 20 min. The reaction was stopped by adding 40 μL of stop buffer (0.25 M EGTA, 0.25 M EDTA). Chromatin-free (naked) DNA was separated from the isolated nuclei and treated with 1 unit of DNase I for 2 min. DNA was extracted and purified as described above, resuspended in 20 μL of Tris-EDTA, and digested with *Bgl*I to create reference-sized DNA fragments. The DNA was separated on 0.8% (w/v) agarose gels, blotted, and hybridized as described above with a probe specific for the *bar*-coding region (probe C; Fig. 4B).

LITERATURE CITED

Assaad FF, Tucker KL, Signer ER (1993) Epigenetic repeat-induced gene silencing (RIGS) in *Arabidopsis*. Plant Mol Biol **22**: 1067–1085

Atkinson RG, Bieleski LRF, Gleave AP, Janssen B-J, Morris BAM (1998) Post-transcriptional silencing of chalcone synthase in petunia using a geminivirus-based episomal vector. Plant J **15**: 593–604

Cheng M, Fry JE, Shengzhi P, Huaping Z, Hironaka CM, Duncan DR, Conner TW, Wan Y (1997) Genetic transformation of wheat mediated by *Agrobacterium tumefaciens*. Plant Physiol **115**: 971–980

Cone K (1989) Yet another rapid plant DNA prep. Maize Gen Coop Newslett **63**: 68

De Block M, Debrouwer D, Moens T (1997) The development of a nuclear male sterility system in wheat: expression of the *barnase* gene under the control of tapetum specific promoters. Theor Appl Genet **95**: 125–131

Fedoroff NV, Chandler V (1994) Inactivation of maize transposable elements. *In* J Paszkowski, ed, Homologous Recombination and Gene Silencing in Plants. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 349–385

Hamilton RH, Kunsch U, Temperli A (1972) Simple rapid procedures for the isolation of tobacco leaf nuclei. Anal Biochem **49**: 48–57

Hansen G, Chilton M-D (1996) "Agrolistic" transformation of plant cells: integration of T-strands generated *in planta*. Proc Natl Acad Sci USA **93**: 14978–14983

Hiei Y, Ohta S, Komari T, Kumashiro T (1994) Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. Plant J **6**: 271–282

Holmes-Davis R, Comai L (1998) Nuclear matrix attachment regions and plant gene expression. Trends Plant Sci **3**: 91–97

Ishida Y, Saito H, Ohta S, Hiei Y, Komari T, Kumashiro T (1996) High efficiency transformation of maize (*Zea mays* L.) mediated by *Agrobacterium tumefaciens*. Nat Biotechnol **14**: 745–750

Iyer LM, Kumpatla SP, Chandrasekharan MB, Hall TC (2000) Transgene silencing in monocots. Plant Mol Biol **43**: 323–346

Koprek T, McElroy D, Louwerse J, Williams-Carrier R, Lemaux PG (2000) An efficient method for dispersing *Ds* elements in the barley genome as a tool for determining gene function. Plant J **24**: 253–264

Kumpatla SP, Chandrasekharan MB, Iyer LM, Li G, Hall TC (1998) Genome intruder scanning and modulation systems and transgene silencing. Trends Plant Sci **3**: 97–104

Liu Y-G, Mitsukawa N, Oosumi T, Whittier RF (1995) Efficient isolation and mapping of *Arabidopsis thaliana* T-DNA insert junctions by thermal asymmetric interlaced PCR. Plant J **8**: 457–463

Matzke AJ, Matzke MA (1998) Position effects and epigenetic silencing of plant transgenes. Curr Opin Plant Biol **1**: 142–148

McElroy D (1999) Moving agbiotech downstream. Nat Biotechnol **17**: 1071–1074

McElroy D, Louwerse JD, McElroy SM, Lemaux PG (1997) Development of a simple transient assay for Ac/Ds activity in cells of intact barley tissue. Plant J **11**: 157–165

Meissner R, Chague V, Zhu Q, Emmanuel E, Elkind Y, Levy AA (2000) A high throughput system for transposon tagging and promoter trapping in tomato. Plant J **22**: 265–274

Meyer P, Heidmann I (1994) Epigenetic variants of a transgenic petunia line show hypermethylation in transgene DNA: an indication for specific recognition of foreign DNA in transgenic plants. Mol Gen Genet **243**: 390–399

Meyer P, Linn F, Heidmann I, Meyer H, Niedenhof I, Saedler H (1992) Endogenous and environmental factors influence 35S promoter methylation of a maize *A1* gene construct in transgenic petunia and its color phenotype. Mol Gen Genet **231**: 345–352

Muskens MWM, Vissers APA, Mol JNM, Kooter JM (2000) Role of inverted DNA repeats in transcriptional and post-transcriptional gene silencing. Plant Mol Biol **43**: 243–260

Parinov S, Sevugan M, Ye D, Yang W-C, Kumaran M, Sundaresan V (1999) Analysis of flanking sequences from *Dissociation* insertion lines: a database for reverse genetics in *Arabidopsis*. Plant Cell **11**: 2263–2270

Park SH, Pinson SR, Smith RH (1996) T-DNA integration into genomic DNA of rice following *Agrobacterium* inoculation of isolated shoot apices. Plant Mol Biol **32**: 1135–1148

Pawlowski WP, Torbert KA, Rines HW, Somers DA (1998) Irregular patterns of transgene silencing in allohexaploid oat. Plant Mol Biol **38**: 597–607

Pröls F, Meyer P (1992) The methylation patterns of chromosomal integration regions influence gene activity of transferred DNA in *Petunia hybrida*. Plant J **2**: 465–475

Que Q, Wang H-Y, English JJ, Jorgensen RA (1997) The frequency and degree of co-suppression by sense chalcone synthase transgenes are dependent on transgene promoter strength and are reduced by premature nonsense codons in the transgene coding sequence. Plant Cell **9**: 1357–1368

Spiker S, Thompson WF (1996) Nuclear matrix attachment regions and transgene expression in plants. Plant Physiol **110**: 15–21

Srivastava V, Anderson OD, Ow DW (1999) Single-copy transgenic wheat generated through the resolution of complex integration patterns. Proc Natl Acad Sci USA **96**: 11117–11121

Stam M, De Bruin R, Kenter S, Van Der Hoorn RAL, Van Blokland R, Mol JNM, Kooter JM (1997a) Posttranscriptional silencing of chalcone synthase in *Petunia* by inverted transgene repeats. Plant J **12**: 63–82

Stam M, Mol JNM, Kooter JM (1997b) The silence of genes in transgenic plants. Ann Bot **79**: 3–12

Sun FL, Elgin SC (1999) Putting boundaries on silence. Cell **99**: 459–462

Tingay S, McElroy D, Kalla R, Fieg S, Wang M, Thornton S, Brettell R (1997) *Agrobacterium tumefaciens*-mediated barley transformation. Plant J **11**: 1369–1376

van Blokland R, Ten Lohuis M, Meyer P (1997) Condensation of chromatin in transcriptional regions of an inactivated plant transgene: evidence for an active role of transcription in gene silencing. Mol Gen Genet **257**: 1–13

Wan Y, Lemaux PG (1994) Generation of large numbers of independently transformed fertile barley plants. Plant Physiol **104**: 37–48

Waterhouse PM, Graham MW, Wang MB (1998) Virus resistance and gene silencing in plants can be induced by simultaneous expression of sense and antisense RNA. Proc Natl Acad Sci USA **95**: 13959–13964

Wirtz U, Osborne B, Baker B (1997) *Ds* excision from extrachromosomal geminivirus vector DNA is coupled to vector DNA replication in maize. Plant J **11**: 125–135

Ye F, Signer ER (1996) RIGS (repeat-induced gene silencing) in *Arabidopsis* is transcriptional and alters chromatin configuration. Proc Natl Acad Sci USA **93**: 10881–10886