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Irregular patterns of transgene silencing in allohexaploid oat

Wojciech P. Pawlowski[1], Kimberly A. Torbert[1], Howard W. Rines[2] and David A. Somers[1,*]
[1]*Department of Agronomy and Plant Genetics, University of Minnesota, 411 Borlaug Hall, 1991 Upper Buford Circle St. Paul, MN 55108, USA (*author for correspondence);* [2]*Plant Science Research Unit, USDA-ARS, St. Paul, MN 55108, USA*

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Abstract

An irregular pattern of transgene silencing was revealed in expression and inheritance studies conducted over multiple generations following transgene introduction by microprojectile bombardment of allohexaploid cultivated oat (*Avena sativa* L.). Expression of two transgenes, *bar* and *uidA*, delivered on the same plasmid was investigated in 23 transgenic oat lines. Twenty-one transgenic lines, each derived from an independently selected transformed tissue culture, showed expression of both *bar* and *uidA* while two lines expressed only *bar*. The relationship of the transgenic phenotypes to the presence of the transgenes in the study was determined using (1) phenotypic scoring combined with Southern blot analyses of progeny, (2) coexpression of the two transgenic phenotypes since the two transgenes always cosegregated, and (3) reactivation of a transgenic phenotype in self-pollinated progenies of transgenic plants that did not exhibit a transgenic phenotype. Transgene silencing was observed in 19 of the 23 transgenic lines and resulted in distorted segregation of transgenic phenotypes in 10 lines. Silencing and inheritance distortions were irregular and unpredictable. They were often reversible in a subsequent generation of self-pollinated progeny and abnormally segregating progenies were as likely to trace back to parents that exhibited normal segregation in a previous generation as to parents showing segregation distortions. Possible causes of the irregular patterns of transgene silencing are discussed.

Introduction

Microprojectile bombardment remains the most successful and general method for genetic transformation of a number of commercially important crop species [4]. Transgenes introduced into plants by microprojectile bombardment are generally inherited as single Mendelian factors (see [19] for review). In most transgenic plants, the introduced DNA becomes stably and permanently integrated into the nuclear genome [19]. However, transgene expression remains largely unpredictable in most transformation experiments.

Several factors related to integration and structure of transgene DNA, such as the number of transgene copies, position in the genome, and methylation, may greatly influence expression of transgenes. Transgene silencing, usually defined as inactivation of transgene expression despite the presence of an unchanged, but possibly methylated, transgene sequence in the plant genome, was initially described in model plants such as tobacco and *Arabidopsis* transformed by *Agrobacterium tumefaciens*. More recently, transgene silencing has been observed in transgenic plants produced by particle bombardment and in economically important crop species [5, 14, 24, 29, 33, 34] although it has been seldom studied in detail [14]. Several hypotheses have been proposed to explain transgene silencing [7, 15]. Some reports associate transgene silencing phenomena with specific features of the transgene integration pattern, e.g., presence of multiple transgene

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copies at the integration locus [15]. Plants transformed using particle bombardment generally exhibit greater variation in transgene copy numbers and have more rearranged transgene copies than plants transformed using *A. tumefaciens* [19]. The higher incidence of multiple transgene copies may cause transgene silencing to be more frequent in plants transformed by particle bombardment than in plants transformed by *Agrobacterium*. Abnormal expression of transgenes can, in many instances, be detected in the first generation of transgenic plants. However, in some experiments, transgene silencing is not observed until large-scale field trials [2]. For effective utilization of genetically engineered germplasm for crop improvement, stable expression of transgenes over many generations and the ability to fix the transgenic phenotype in homozygous lines are crucial. Therefore, long-term studies are needed that analyze expression and inheritance of transgenes through several generations of genetically engineered plants. Transgene expression and inheritance has been described in advanced generations of transgenic maize [1, 24], tobacco [21] and common bean [26].

Transformation of allohexaploid oat (*Avena sativa* L.) using microprojectile bombardment was first reported by Somers *et al.* [27]. To assess the usefulness of the transformation system for oat improvement, we investigated the expression and inheritance of transgenic phenotypes in transgenic oat plants. In this study, transgenic phenotypes encoded by two linked transgenes, *bar* and *uidA*, were analyzed in 23 oat lines produced from independent transformation events. Twenty-one of these lines exhibited expression of both transgenic phenotypes. Expression and inheritance of the transgenic phenotypes were determined in up to four generations of progeny produced from the primary transgenic plants (T_0). Nearly 50% of the transgenic lines exhibited inheritance of the GUS phenotype (encoded by *uidA*) that fit Mendelian single-gene segregation ratios. However, transgene silencing frequently caused distorted segregation of the transgenic phenotype in the remaining lines and a lack of coexpression of the two transgenic phenotypes, GUS-staining and resistance to PPT-containing herbicides (encoded by *bar*) in the majority of the transgenic lines. Transgene silencing and distortions of transgene inheritance caused by transgene silencing were unstable and often reversible in a following generation after self-pollination. Here we describe the specific patterns of silencing observed and discuss possible causes of irregular patterns of transgene silencing.

Materials and methods

Transgenic oat plants

Transgenic oat plants were produced by microprojectile bombardment of oat tissue cultures initiated from F_4 embryos of the genotype GAF-30/Park [25] with the plasmid pBARGUS [9] (Figure 1) as described by Somers *et al.* [27]. The plasmid supplied the selectable marker gene *bar* from *Streptomyces hygroscopicus* [31] coding for a herbicide-detoxifying enzyme phosphinothricin (PPT) acetyltransferase (PAT) and the reporter *uidA* gene from *Escherichia coli* coding for β-glucuronidase (GUS) [13]. Some transgenic lines, in addition to pBARGUS, were cotransformed with either the plasmid pH24, which is similar to pCaMV-NEO [8] but contains a CaMV 35S promoter/*Adh1* intron/*nptII* construct, or one of three plasmids, pMAV, pPAV and pRPV, containing coat protein genes of three different strains of barley yellow dwarf virus (BYDV) [16] (Figure 1). The BYDV coat protein genes were placed under the control of the CaMV 35S promoter and ligated into pGEM3z (Promega, Madison, WI). The presence and expression of these plasmids cotransformed with pBARGUS is not addressed in this study. The BYDV coat protein cotransformants have been described [16]. All transgenic plants were grown in containment in controlled-environment growth chambers [32].

Transgene phenotypic assays

Kernels were cut in half; the endosperm portion of each kernel was stained for GUS activity using the histochemical assay [13] while the portion containing the embryo was planted for further plant analysis. The GUS stain solution [13] was adjusted to pH 8.0 to reduce non-specific background staining. The samples were incubated in the stain for two days at 30 °C before scoring for GUS activity.

Expression of PAT, encoded by the *bar* gene, was determined by screening transgenic plants for resistance to the herbicide PPT [31]. A 0.2% aqueous solution of PPT in the form of glufosinate ammonium (Riedel-de Haën, Seelze, Germany) was applied with a cotton swab on a 1 cm wide leaf segment of plants just before emergence of the flag leaf. Resistance to the herbicide was scored after seven days.

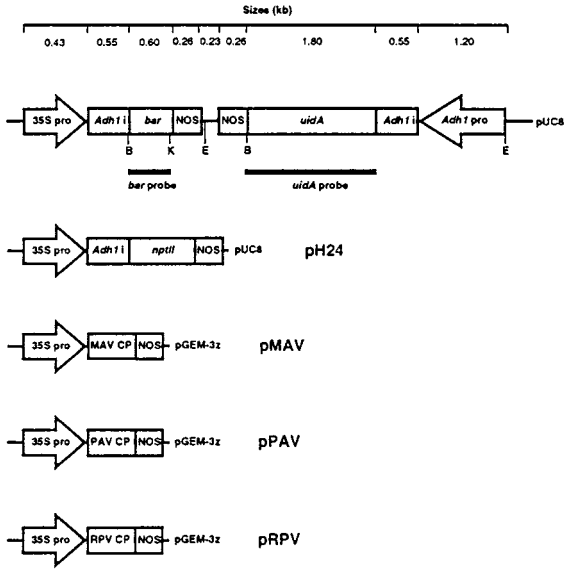

Figure 1. Restriction maps of the plasmids (not drawn to scale) used for oat transformation. Positions of probes used in Southern blot analyses are indicated by black bars underneath the appropriate sequences. Abbreviations: 35S pro, CaMV 35S promoter; *Adh1* pro, promoter of the maize *Adh1* gene; *Adh1* i, first intron of the maize *Adh1* gene; CP, barley yellow dwarf virus coat protein gene; NOS, *nos* gene termination sequence; pUC8, pGEM-3z, plasmid backbones. Restriction sites: B, *Bam*HI; E, *Eco*RI; K, *Kpn*I.

Segregation analyses

Transgenic plants were allowed to self-pollinate to produce subsequent generations of progeny. Primary transgenic plants (T_0 generation) that were regenerated from a single transgenic tissue culture and progeny of the primary transgenics were designated as a 'transgenic line'. Thus, each transgenic line traced back to a different transgenic tissue culture and represented an independent transformation event. Segregation ratios of GUS^+ and GUS^- seed were analyzed statistically with the χ^2 test for goodness of fit at $P < 0.05$ with the Yates' correction for continuity where appropriate [30]. Seeds harvested from 1 to 63 plants (on average 11) were analyzed by GUS staining in each transgenic line in each of the generations tested. On average, 20 seeds were stained per plant. For the PPT resistance analyses, an average of 30 plants were screened per transgenic line.

DNA analyses

DNA was extracted from plant leaf tissue following the procedure described by Hu and Quiros [11]. About 30 μg of DNA was digested overnight with restriction endonucleases and separated on 1.0% agarose gels. The DNA was transferred from the gel onto Immobilon N membrane (Millipore, Bedford, MA) as described by Southern [28]. The presence of transgenes was detected using a 1.8 kb *Bam*HI/*Sst*I *uidA*-containing fragment from pBI221 [13] and a 536 bp *bar*-containing *Bam*HI/*Kpn*I fragment from pBARGUS as probes (Figure 1). Double-stranded DNA probes were ^{32}P-labeled with the Rediprime (Amersham Life Science, Arlington Heights, IL) or the Prime-a-gene (Promega, Madison, WI) kits. Hybridization buffer consisted of 5× SSPE (0.75 M sodium chloride, 0.05 M sodium phosphate, 5 mM EDTA), 5× Denhardt's reagent (0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin), 5% dextran sulfate, 0.5% SDS and 1 mg/ml of denatured salmon sperm DNA. Hybridization was carried out at 65 °C overnight and all subsequent washes were performed according to Hosaka *et al.* [10] except for the omission of sodium pyrophosphate from the wash solution. Membranes were analyzed by autoradiography using X-Omat AR5 film (Eastman Kodak, Rochester, NY).

Results

Integration of uidA and bar in transgenic oat lines

Twenty-three transgenic oat lines, each representing an independent transformation event, were produced by microprojectile bombardment. The transgenic lines 300 and 301 were transformed with pBARGUS (Figure 1). The remaining 21 transgenic lines, in addition to being transformed with pBARGUS, were cotransformed with either the plasmid pH24 or one of three plasmids containing coat protein (CP) genes of barley yellow dwarf virus (BYDV) [16] (Figure 1). Detailed Southern blot analyses, including reconstruction of *bar* and *uidA* transgene copy numbers, were performed on regenerated plants from 19 of the 23 lines (Table 1). They indicated that the integration pattern of transgene DNA in the oat genome was complex as shown for the *uidA* gene in Figure 2. All 19 lines exhibited a pBARGUS restriction fragment of the expected size hybridizing to the *bar* probe, hereafter referred to as 'full-length transgene' (data not shown). Eighteen lines exhibited the full-length *uidA* transgene (pBARGUS restriction fragment of the expected size hybridizing to the *uidA* probe). Additional fragments hybridizing to the *uidA*- and *bar*-specific probes, both

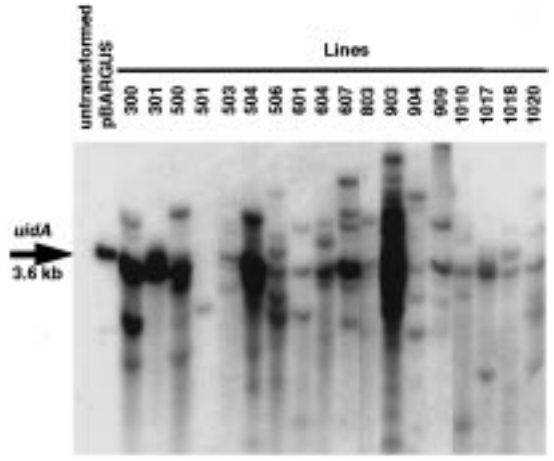

Figure 2. Examples of complex integration patterns of transgene DNA in 18 transgenic oat lines. DNA was restricted with *Bam*HI and *Eco*RI and hybridized with the *uidA* probe. 3.6 kb is the expected length for a pBARGUS restriction fragment containing *uidA*.

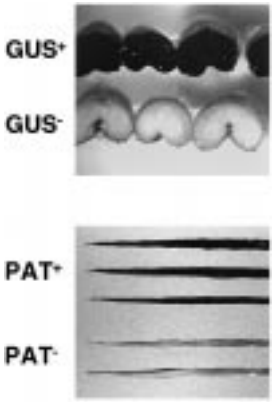

Figure 3. Scoring of the GUS and PAT transgenic phenotypes in genetically engineered oat plants.

smaller and larger than the full-length transgenes, were detected in the majority of lines including line 501 which did not have a full-length *uidA* transgene (Table 1).

Analysis of segregation of transgene integration patterns in progenies of each transgenic line was conducted to determine the number of transgene loci. In 18 of the 19 transgenic lines analyzed by Southern blot analyses, *bar*- and *uidA*-hybridizing transgene fragments cosegregated as a single genetic locus (on average, 18 progeny plants were tested per line; data not shown). The presence of a single transgene locus was confirmed by probing Southern blots with the entire pBARGUS sequence, which also hybridized to sequences in the cotransformed plasmids in common with pBARGUS, i.e. 35S promoter and NOS 3′ sequences of pH24, pMAV, pPAV and pRPV (Figure 1). In each of the 18 lines, the transgene-positive progeny within a line exhibited the same transgene integration pattern, which was identical to the pattern observed in the original transgenic tissue cultures. Line 500 exhibited two independent transgene loci based on Southern analysis of segregating progeny.

Cosegregation of transgenes and transgenic phenotypes

GUS staining was tested in the endosperm portion of the caryopsis (Figure 3). Because the endosperm is a progenital tissue, the results of GUS staining indicated the transgenic phenotype of the next generation of progeny. The embryo portion of the caryopsis was planted and Southern blot analyses and analyses of PPT resistance were performed on subsequently produced leaf tissue. GUS staining and PPT-resistance are hereafter referred to as the transgenic phenotypes (Figure 3). The relationship of the transgenic phenotypes to the presence of the *uidA* and *bar* transgenes was investigated by: (1) phenotypic scoring combined with Southern blot analyses of T_1 and/or T_2 progeny, (2) analyses of coexpression of the transgenic phenotypes since the two transgenes cosegregated at the DNA level in 18 of the transgenic lines examined, and (3) reactivation of a transgenic phenotype in progenies of self-pollinated transgenic plants that did not exhibit a transgenic phenotype.

All T_0 plants in all 23 transgenic lines were resistant to PPT. Progeny of 16 lines were examined by Southern blot analyses (not shown) for the *bar* gene in relation to expression of the PPT-resistance phenotype. All PPT-resistant segregants exhibited full-length *bar* gene fragments but PPT resistance cosegregated with the presence of the *bar* gene in only 5 of the 16 lines. Within the remaining 11 lines, some *bar*-positive plants were resistant to PPT but other *bar*-positive progeny were susceptible to PPT even though the *bar* hybridization patterns of the PPT-resistant and -susceptible plants were indistinguishable. These results suggested a high frequency of silencing of the *bar* transgene among these 11 lines.

GUS expression was detected in seed produced on plants from 21 of the 23 lines. Various levels of GUS activity were observed in the seed endosperm in different lines, ranging from very strong (as shown in Figure 3) to very weak. However, the GUS staining intensity was always uniform within the endosperm and variegation of GUS expression was not observed.

Table 1. Transgene copy numbers and rearrangements in 19 transgenic oat lines.

Line	*bar* copies		*uidA* copies	
	full-length[a]	rearrangements[b]	full-length[a]	rearrangements[b]
300	4	2	2	4
301	3	1	1	1
500	2	1	1	4
501	1	0	0	1
503	3	1	1	1
504	5	6	4	6
506	1	4	1	6
601	1	1	1	2
604	2	2	2	5
607	1	0	3	6
803	1	1	1	2
804	2	2	2	4
903	5	3	4	11
904	3	1	1	4
909	1	1	1	4
1010	2	1	1	3
1017	2	1	1	2
1018	1	1	1	2
1020	2	1	1	5

[a] Transgene copy number based on the intensity of hybridization of the *bar* and *uidA* gene-specific probes in copy number reconstruction analysis.
[b]Number of different sized transgene-hybridizing fragments.

Kernels showing at least some GUS activity wwere classified as GUS$^+$. Progeny plants from a subsample of 13 of the 23 GUS-expressing lines were tested for GUS expression in relation to the *uidA* transgene. Restriction fragments that corresponded to the presence of the full-length *uidA* gene were detected in all GUS$^+$ plants. In 6 of the 13 lines, all *uidA* transgene-positive individuals exhibited the GUS staining (Figure 4). Individuals that lacked the GUS phenotype, but had a *uidA* hybridization pattern identical to the GUS$^+$ progeny within the same line, were found in the remaining 7 lines tested implicating transgene silencing (Figure 5). In line, 500 which exhibited two transgene loci, one locus cosegregate with presence of the GUS-staining and PPT-resistance phenotypes. The second locus lacked *bar* and only contained a rearranged restriction fragment of *uidA*, which did not cosegregate with presence of the GUS phenotype and thus was most likely non-functional. Lines 501 and 904 lacked expression of the GUS phenotype, presumably due to rearrangements of the *uidA* gene.

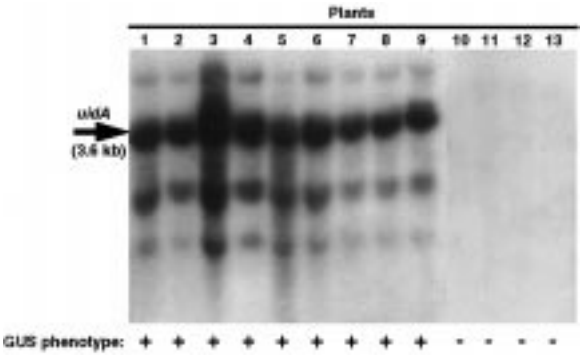

Figure 4. Cosegregation of the GUS phenotype and the *uidA* gene in 13 plants from a T_2 progeny of line 300. Presence or absence of GUS staining is indicated by + and −, respectively. DNA was restricted with *Bam*HI and *Eco*RI and hybridized with the *uidA* probe.

Coexpression of two transgenes

Data for coexpression of the two transgenes in the progeny of the 21 GUS$^+$ and PPT-resistant lines were evaluated to further investigate the frequency of transgene silencing (Table 2). Because Southern blot analyses of the transgenic plants and their progeny indicated

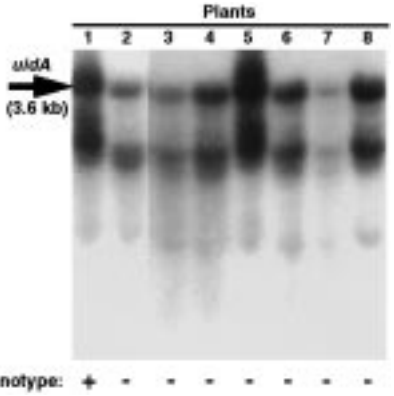

Figure 5. Lack of cosegregation of the GUS phenotype and the *uidA* gene in 8 plants from a T_2 progeny of line 803. Presence or absence of GUS staining is indicated by + and −, respectively. DNA was restricted with *Bam*HI and *Eco* RI and hybridized with the *uidA* probe.

that all introduced plasmid DNA segregated as one genetic locus in all transgenic lines except for line 500, which exhibited two transgene-hybridizing loci, lack of coexpression of the transgenic phenotypes in plants within a line provided further evidence for transgene silencing. In all lines tested at least some individuals exhibiting coexpression of GUS staining and PPT resistance were found, but complete coexpression of the transgenic phenotypes in all transgenic progeny plants tested was found in only 3 of the 21 transgenic lines (Table 2). Seven lines exhibited lack of coexpression of the two transgenes in low numbers of progeny (less than 20% of plants per line). The remaining 11 lines had numerous individuals lacking coexpression of the transgenic phenotypes. The lack of coexpression of the transgenic phenotypes was most often due to the absence of PPT resistance, while the GUS phenotype was expressed in the endosperm: 46% of all GUS^+ plants tested in the experiment showed no PPT resistance. The reverse situation was less frequent: only 17% of the PPT-resistant plants showed no expression of the GUS phenotype. However, in lines 500 and 903 lack of GUS expression was more frequent than lack of PPT resistance (data not shown). Approximately 75% of plants in progenies that were determined to be homozygous for GUS in the 21 lines lacked PPT resistance. Overall, silencing of *uidA* and/or *bar* was detected in 19 of the 21 lines that exhibited expression of both transgenic phenotypes (Table 2). Silencing of either *uidA* or *bar* was most frequent, although plants exhibiting silencing of both transgenes also were found.

Segregation of the GUS phenotype

We further investigated the effects of transgene silencing on inheritance of the GUS phenotype in up to four generations of self-pollinated progeny in most transgenic lines. Ten lines segregated for the GUS phenotype in agreement with Mendelian 3:1 ratios in all generations tested (Tables 2 and 3A). Nine of these 10 lines produced GUS^+ homozygotes identified in GUS progeny tests (Table 2). Ten GUS^+ homozygous T_2 progenies from five lines (300, 804, 809, 1010, and 1017) were advanced to the T_3 generation to examine whether the putative homozygotes were stable in following generations. Of the 10 progenies, 8 produced uniformly GUS^+ offspring while two progenies, from lines 300 and 904, produced some GUS^- plants in the offspring. One of the stably homozygous T_3 progenies from line 300 was advanced further to T_4 and again yielded all GUS^+ seeds. No putative GUS^+ homozygotes were recovered in line 506, which probably resulted from the fact that only two GUS^+ T_1 and one T_2 progeny plants were analyzed in this line.

Line 301 showed no transgene silencing based on southern bloth analysis (data now shown), although it consistently exhibited distorted GUS inheritance ratios and produced no GUS^+ homozygotes. It is likely that reduced transgene transmission to progeny, possibly due to a deleterious mutation linked to the transgene integration event, was responsible for the distorted GUS segregation ratios in this line.

Silencing of *uidA* was detected in 10 transgenic lines that exhibited distorted GUS segregation ratios in all or some of the generations analyzed (Tables 2 and 3B). This observation suggested that *uidA* silencing was the primary cause of the distorted GUS segregation ratios and the generational variation in segregation ratios. Five of the 10 lines (500, 607, 803, 1015 and 1105) exhibited consistent distortions from Mendelian segregation ratios in all generations tested (Table 3B). Five other lines (503, 601, 604, 903 and 909) showed segregation ratios that varied from generation to generation, alternating between Mendelian and non-Mendelian segregation in different generations. Some plants with normally segregating progenies were found in the abnormally segregating lines. Abnormally segregating progenies were as likely to trace back to parents that exhibited normal segregation in a previous generation as to parent plants showing segregation distortions (Figure 6). Moreover, when abnormally segregating progenies were advanced to the next generation, nearly one half of them exhibited

Table 2. Coexpression, inheritance and silencing of the GUS staining and PPT resistance transgenic phenotypes in 23 transgenic oat lines.

Line	Co-expression	Mendelian inheritance of GUS phenotype[a]	GUS^+ homozygotes recovered	Transgene silencing[b]	
				bar	*uidA*
300	−	+	+	+++	−
301	+	−	−	−	−
500	−	−	+	++	+++
501	ne	ne	ne	−	ne
503	−	−	+	+++	++
504	−	+	+	+++	+
506	−	+	−	++	++
601	−	−	+	+	++
604	−	−	+	+++	+
607	−	−	+	++	+
803	−	−	−	++	+++
804	−	+	+	+++	+
809	−	+	+	+	−
810	+	+	+	−	−
903	−	−	+	++	++
904	ne	ne	ne	−	ne
909	−	−	+	+++	++
1010	−	+	+	+++	+
1015	−	−	−	−	+++
1017	−	+	+	++	−
1018	−	+	+	−	+
1020	−	+	+	+++	−
1105	+	−	−	−	++

[a]Mendelian inheritance in all generations tested is denoted by +; non-Mendelian inheritance in all or some of the generations tested is denoted or by −; ne lines completely lacked *uidA* expression.

[b] Frequency of transgene silencing indicated by: − no silencing detected; + <10% of transgene-positive plants exhibiting transgene silencing; ++ transgene silencing detected in >10% but <50% of transgene-positive plants; + + + silencing detected in >50% of transgene-positive plants.

Mendelian GUS segregation ratios while the other half exhibited non-Mendelian inheritance patterns (Figure 6).

Putatively homozygous progeny plants were identified, based on the GUS progeny test, in lines 500, 503, 601, 604, 607, 903, and 909 that exhibited segregation distortion (Table 2). The ratio of GUS^+ homozygotes to hemizygotes varied among the lines from Mendelian 1:2 ratio to fewer homozygotes than expected. For example, in line 500, the ratio of GUS^+ homozygotes to heterozygotes was 2:17 in the T_1 and 6:18 in the T_2 generations. In line 607, a normal number of putative GUS^+ homozygotes was found in T_1 but no putative homozygotes were identified in the T_2 progeny. Sixteen putatively homozygous T_2 progenies from six abnormally segregating lines (500, 503, 601, 604, 607, and 909) were advanced to the T_3 generation. Eleven T_3 progenies were uniformly GUS^+, while in five progenies, from lines 500, 604, and 909, some individuals in the offspring were GUS^-. One stably homozygous T_3 progeny from line 601 was advanced to the T_4 generation and again produced uniformly GUS^+ offspring.

Epigenetic reactivation of the transgenic phenotype

Reactivation of GUS expression as detected by the presence of GUS^+ individuals in the progeny of GUS^- plants was observed in 13 of the 21 lines that exhibited GUS expression. Such progenies were infrequent in 9 of these 13 lines. However, in lines 500,

Table 3. Examples of transgenic oat lines exhibiting different types of segregation of the GUS phenotype. T_1 seed produced on regenerated transgenic plants (T_0) were planted for T_2 progeny tests. T_2 seed from presumed heterozygotes were progeny tested in the T_3 generation.

Line	Generation	Deduced genotype[b]	Number of plants tested	Number of seeds		$\chi 2$[a]
				GUS$^+$	GUS$^-$	
A. Mendelian segregation						
1017	T_1	*uidA*$^+$/*uidA*$^-$	3	54	17	0.04
	T_2	*uidA*$^+$/*uidA*$^+$	1	7	0	–
		uidA$^+$/*uidA*$^-$	2	61	15	1.12
		uidA$^-$/*uidA*$^-$	3	0	20	–
	T_3	*uidA*$^+$/*uidA*$^+$	2	26	0	–
		uidA$^+$/*uidA*$^-$	6	79	35	1.98
		uidA$^-$/*uidA*$^-$	1	0	18	–
B. Non-Mendelian segregation of the GUS phenotype in all generations						
803	T_1	*uidA*$^+$/*uidA*$^-$	1	31	67	96.00*
	T_2	*uidA*$^+$/*uidA*$^+$	0	–	–	–
		uidA$^+$/*uidA*$^-$	5	39	46	36.90*
		uidA$^-$/*uidA*$^-$	8	0	105	–
	T_3	*uidA*$^+$/*uidA*$^+$	0	–	–	–
		uidA$^+$/*uidA*$^-$	7	13	115	283.59*
		uidA$^-$/*uidA*$^-$	3	0	52	–

* Segregation ratio significantly different from 3 : 1 at $P < 0.05$.
[a] χ^2 values were calculated for deviations from 3 : 1 segregation ratio at $P = 0.05$ using Yates' correction for continuity where appropriate.
[b] Hypothetical genotype was assigned based on phenotypic segregation in the progeny.

503, 506, and 803 more than 50% of GUS$^-$ plants produced GUS$^+$ progenies. Of 52 'reactivated' progenies, 19 segregated according to the Mendelian 3:1 ratio, 24 exhibited non-Mendelian segregation ratios with fewer GUS$^+$ individuals than expected for Mendelian segregation ratios and 9 appeared to be GUS$^+$ homozygotes. To further investigate the frequency of epigenetic reactivation, the presence of the GUS phenotype was studied in 22 transgene-positive progenies produced from GUS$^-$ plants from lines 500 and 803. The GUS phenotype was detected in 16 progenies; five progenies (all from line 500) exhibited segregation of the GUS phenotype that fit the Mendelian 3:1 ratio, eight progenies (three from line 500 and five from line 803) exhibited non-Mendelian segregation ratios, and three progenies (all from line 500) appeared to be GUS$^+$ homozygotes. Transgene reactivation did not occur in eight progenies (seven from line 500 and one from line 803). These observations indicated that while transgene expression was reactivated in some of the next generation progenies, in other progenies transgene silencing persisted resulting in lack of the GUS phenotype or abnormal GUS segregation ratios.

Discussion

Single-locus inheritance of transgenes is the most frequent pattern of inheritance reported in plants produced by microprojectile bombardment [1, 5, 18, 23, 24, for review see 19]. Genetic and molecular analyses of transgenic oat plants suggested the presence of single transgene loci in 22 of the 23 transgenic oat lines analyzed. In line 500, a second, independently segregating, and non-expressed transgene locus was identified in addition to the transgene locus containing the functional *bar* and *uidA* transgenes. Transgene silencing was detected in 19 of the 21 lines expressing both transgenic phenotypes (Table 2). Transgene silencing

manifested itself by (1) lack of the transgenic phenotype in plants shown to have the transgenes in Southern blot analyses, (2) lack of coexpression of the GUS and PPT-resistance phenotypes despite the fact that the two transgenes always cosegregated at the DNA level in transgenic plants, (3) distorted segregation of the GUS phenotype in some transgenic lines, and (4) reactivation of the transgenic phenotypes in self-pollinated progenies of GUS^- or PPT-susceptible plants. Ten of the 21 GUS^+ transgenic lines exhibited inheritance ratios of the GUS transgenic phenotype that consistently fit Mendelian segregation ratios in up to three generations of progeny. Transgene silencing presumably accounted for the significantly distorted ratios of inheritance of transgenic phenotypes in the remaining 11 lines. Putative GUS^+ homozygotes were identified based on the phenotype of their progeny in 9 of the 10 lines showing Mendelian transgene inheritance and in 7 of the 11 lines that exhibited segregation distortions. Most of the homozygous progenies appeared stable when advanced to a subsequent generation, including those produced from lines showing abnormalities of transgene inheritance. In some lines that exhibited abnormal segregation ratios but produced putative homozygotes based on progeny tests, homozygous individuals were only recovered in some generations and the ratio of phenotypically deduced homozygotes to hemizygotes was lower than the ratio expected based on Mendelian principles of inheritance. Only one transgenic oat line exhibited Mendelian segregation of the GUS phenotype and perfect cosegregation of both transgenes and the transgenic phenotypes indicating that the oat transformation system will be useful for crop improvement but that many independent events must be produced to overcome the high frequency of transgene silencing.

Transgene silencing has been extensively documented in transgenic plants produced by *Agrobacterium*-mediated transformation and direct DNA uptake into protoplasts (for review see [7, 15]) and is also found in microprojectile bombardment experiments [5, 14, 24, 29, 33, 34]. The two physically linked transgenes delivered on the same pBARGUS plasmid exhibited different patterns of expression and silencing. Lack of coexpression of transgenic phenotypes encoded by the two genes was observed in plants from 18 of the 21 lines examined. Overall, silencing of *bar* was more frequent than silencing of *uidA* in terms of the number of lines and individuals affected. Although silencing has often been associated with the presence of multiple transgene copies [3, 15], the presence and frequency of transgene silencing in transgenic oat did not appear to be associated with the number of copies of the structural genes (Tables 1 and 2). However, the additional CaMV 35S promoter copies from the cotransformed BYDV-CP plasmids (Figure 1) could be responsible for a higher frequency of *bar* silencing if silencing resulted from the presence of multiple transgene copies. Lack of PPT resistance was observed in 75% of the plants in progenies homozygous for the GUS phenotype, indicating that a copy number-dependent mechanism of silencing may have contributed to the silencing of *bar*. Another plausible explanation for a higher frequency of *bar* silencing may be that the CaMV 35S promoter regulating the expression of *bar* was intrinsically more prone to silencing than the maize *Adh1* promoter regulating the expression of *uidA*. Different levels of expression of linked genes are not uncommon in transgenic plants, even if the transgenes are almost identical [22]. Contrary to our results, several authors have reported that expression of the selectable marker gene (*bar*) was more stable than expression of a linked unselected gene (*uidA*) in transgenic maize, rice, and wheat [5, 24, 29, 34], although different promoters were used in some of these studies.

Transgene silencing and distortions of transgene inheritance were highly unstable in transgenic oat. Transgene expression was frequently reactivated in progenies of the plants that exhibited transgene silencing. Distortions of transgene inheritance also were reversible in self-pollinated progeny of some transgenic oat lines (Figure 6). Individual progenies that exhibited abnormal inheritance were found in four transgenic oat lines with apparently normal inheritance and, similarly, normally segregating progenies were found in 10 of the 11 abnormally segregating lines. These results indicated that the difference between the lines segregating normally and abnormally was quantitative rather than qualitative and reflected only different frequencies of plants producing normally and abnormally segregating progenies in the two types of lines. The instability of the transgene silencing state suggested a triggering mechanism controlling transgenic silencing. This mechanism could be controlled by endogenous loci segregating in the progeny of the primary transgenic plants, similar to the ones described by Dehio and Schell [6] in *Arabidopsis*. However, this possibility is not likely in transgenic oat because the tissue cultures used for oat transformation were initiated from F_4 embryos and the probability of a specific locus segregating in several transgenic lines

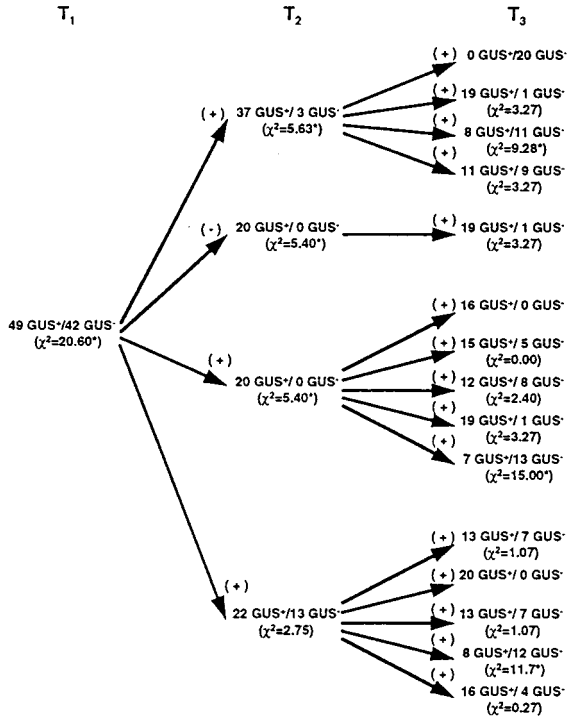

Figure 6. Examples of variation of segregation ratios of the GUS phenotype in line 604. Numbers of GUS^+, GUS^- seeds and χ^2 at $P = 0.05$ are given for each progeny. Presence or absence of GUS staining in each caryopsis that gave rise to a progeny are indicated in parenthesis by $+$ or $-$. Not all progenies in the line are presented. * segregation ratio significantly different from 3:1 at $P < 0.05$.

tracing back to different F_4 embryos was low unless the heterozygosity was produced by tissue culture-induced variation [20]. Alternatively, the instability of transgene silencing may be caused by an unlinked fragment of transgene DNA (e.g., a piece of the CaMV 35S promoter) acting as a silencer [15]. However, all introduced plasmid DNA detected in Southern blot analyses including the CaMV 35S promoter fragments segregated as a single genetic locus in all lines, except line 500, ruling out this possibility. Finally, the instability of the silencing state could be caused by the structure of the transgenic locus itself. A non-heritable 'epiallele' of the transgenic locus leading to a semi-dominant transgene silencing state when homozygous was described in tobacco by Iglesias *et al.* [12]. However, the irregular patterns of transgene silencing and reactivation of transgene expression in oat suggested a random epigenetic process.

In this report, we documented the occurrence of silencing in 19 of 21 of transgenic oat lines in which expression of the GUS and PPT-resistance phenotypes was tested. It has been suggested that a general gene silencing mechanism might be responsible for limiting expression of additional copies of genes in polyploids to protect cells from excessive levels of gene expression [15] and that an increase in ploidy level can trigger silencing [17]. Hexaploid oat may possess a strong mechanism preventing unwanted gene expression; as few as two copies of any transgenic sequence could be sufficient to trigger a transgene silencing response. We frequently observed two or more copies of the *bar* and *uidA* genes (Table 1). Because of the large size of the oat genome and the small percentage of highly expressed sequences in the genome, most transgenes may have integrated into non-expressed or low-expressed genomic regions prone to chromatin condensation, methylation and gene silencing. Data on transgene expression and silencing are still scarce in other polyploid species with large genomes. However, it could be expected that a high frequency of transgene silencing also might be encountered in these species. A further confounding factor is that oat transformation depends on a tissue culture system and thus the effects of tissue culture-induced variation [20] should not be overlooked. However, whether or how these factors may have affected transgene silencing is not clear. The high frequency of transgene silencing and distorted transgene inheritance encountered in oat in this study suggests that a system stabilizing transgene expression will be beneficial in future oat transformation endeavors.

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