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Single-copy transgenic wheat generated through the resolution of complex integration patterns

(wheat transformation/site-specific recombination/Cre-*lox*)

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ABSTRACT **Genetic transformation of plants often results in multiple copies of the introduced DNA at a single locus. To ensure that only a single copy of a foreign gene resides in the plant genome, we used a strategy based on site-specific recombination. The transformation vector consists of a transgene flanked by recombination sites in an inverted orientation. Regardless of the number of copies integrated between the outermost transgenes, recombination between the outermost sites resolves the integrated molecules into a single copy. An example of this strategy has been demonstrated with wheat transformation, where four of four multiple-copy loci were resolved successfully into single-copy transgenes.**

The production of transgenic crop plants is an expanding component of agricultural biotechnology. For commercial success, it will be crucial that the introduced traits be transmitted faithfully through successive generations in a predictable manner. Unfortunately, this does not always hold true, as transgene inactivation is becoming a frequently observed phenomenon. Though not well understood, certain factors affecting transgene inactivation have been described that include multiple-copy integration (1–3), differential base composition between transgene and the integration site (4), hyperexpression of transgenes (5, 6), and environmental factors (7). Although single-copy transgenes can be silenced (5, 7), there is a much higher incidence of gene instability correlated with high transgene copy number (1–3, 8). Complex integration sites undergo either structural instability such as intrachromosomal recombination between multiple copies, sometimes resulting in loss of the transgene (1, 9, 10), or chemical modification such as DNA methylation (11).

Potential means to ensure the stability of a transgene include insulating the transgene with matrix-attachment regions (4), avoiding repetition of promoter or transgene sequences, particularly inverted repeats (12), and using moderate promoters to avoid hyperexpression (8, 13). For proper implementation of these features, it would be critical to start with a single, intact copy of the DNA. The conventional method to achieve this aim is to screen among the pool of transformants (surveyed in ref. 8). However, the occurrence of plants containing a single, intact transgene in cereals is rare (14, 15). In the past 7 years, there have been more than 14 independent reports of wheat transformation (15–27). Based on DNA hybridization data of the analyzed plants, less than 10 are potential single-copy integrants (8 of 85 biolistic transformants and ≈1–2 of 26 Agrobacterium-mediated transformants). Because of low transformation efficiencies, obtaining transgenic wheat is, by itself, a challenging task. If the goal is to achieve single-copy lines, then the task becomes an order of magnitude greater.

Different approaches have been used to enrich for single-copy transgenic lines. An "Agrolistic" method has been described based on cobombarding *VirD* genes along with T-DNA borders flanking the introduced transgene. The resulting transgenic maize calli were found to contain one to two transgene copies at 10–35% efficiency. However, the cointegration of *VirD* DNA is a frequent event (28, 29). In another approach, the treatment with niacinamide, which is believed to be an inhibitor of recombination, yielded single-copy wheat lines at ≈8% efficiency (15). To achieve near-100% recovery of single-copy lines, we designed a strategy in which every multicopy locus can be converted to a single-copy state. Concomitant with this conversion is the removal of the marker gene. Selectable markers are not necessary after transformation, and their presence precludes using the same selection scheme for subsequent transformations (30, 31). Moreover, one preliminary report attributed a reduction in agronomic performance to the presence of the selectable marker.¶ In this report, we describe generating single-copy, marker-free transgenic wheat lines from each of four different complex-integration loci.

MATERIALS AND METHODS

Transformation Vectors. Plasmids were constructed by standard recombinant DNA methods and contain pUC18 backbones. The Cre-*lox* system consists of the 38-kDa Cre recombinase that recognizes a 34-bp target sequence known as *lox* (32, 33). Various *lox* alleles have been described (34, 35). The 7-kb plasmid pVS11 (Fig. 1*A*) contains a fragment flanked by synthetic *lox511* alleles of opposite orientation in pUC18. Within this fragment lies a *bar*-coding region flanked by *loxP* sites in direct orientation. Upstream and downstream of *bar* are, respectively, the rice actin promoter (36), denoted as *P1*, and the maize ubiquitin promoter (37), denoted as *P2*. Although not relevant to this report, an *FRT* site (FLP-*FRT* recombination system) is inserted upstream of *P1* (not shown in the figures), intended for use in future recombination experiments. Plasmids pP2-cre (6.4 kb) and pP2-bar (5.5 kb) are also described in Fig. 1*A*.

Wheat Transformation. Immature embryos, collected from greenhouse or field-grown Spring wheat (var. Bobwhite), were transformed by particle bombardment (38) with pVS11 or by equal amounts of pP2-cre and pP2-bar. Bombarded embryos were cultured and selected in the presence of bialaphos (3 mg/liter). Putative transformants were grown in the green-

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¶Hucl, P, Caswell, K., Demeke, T. & Chibbar, R. N. (1998) Ninth International Wheat Genetics Symposia, August 2–7, 1998, Vol. 3, pp. 189-191.

house for molecular analyses. Cre activity in pP2-cre-transformed plants was determined by bombarding T1 embryos with pHK52, which exhibits β-glucuronidase (GUS) activity upon Cre-mediated inversion of the *gus* coding region. The embryos were stained for GUS activity 48 hr after bombardment.

Molecular Analyses. Wheat genomic DNA (10 μg), isolated by using the cetyltrimethylammonium bromide method, was subjected to Southern hybridization with ^{32}P-labeled DNA probes. Recombination junctions in the wheat genome were detected by PCR analysis by using primers specific to *P1* (5′-CAGCATTGTTCATCGGTAG-3′) and *P2* (5′-AGGCTGGCATTATCTACTCG-3′). Intermolecular Cre-*lox* recombination reactions between PCR-derived junctions and *lox*-containing DNA fragments were conducted *in vitro* as described (34). DNA sequencing of PCR fragments was carried out by using the Applied Biosystems Prism automated-sequencing system.

RESULTS AND DISCUSSION

General Strategy. The transformation vector pVS11 contains a DNA fragment flanked by recombination sites in opposite orientations (Fig. 1*A*). The *lox* allele used for the outermost sites is known as *lox511*, a mutant variant of the wild-type *loxP* sequence (35). Regardless of the number of copies integrated between the outermost transgenes, and the relative orientation of the outermost pVS11 fragments, recombination between the outermost *lox511* sites will resolve multiple units into a single copy. A second set of recombination sites is used for removal of the selectable marker gene, *bar*, that encodes resistance to the herbicide bialaphos. This second set of *loxP* sites flanks *bar* in the same orientation. Because *lox511* does not recombine with *loxP*, two independent recombination reactions are expected to occur. The *lox511*-to-*lox511* recombination will delete internal copies of the transformation DNA to produce a single unit insertion, and the *loxP*-to-*loxP* recombination will excise the marker gene. The expected result would be a marker-free, single-copy insertion of the *lox511-P1-loxP-P2-lox511* fragment at the integration locus, where *P1* and *P2* indicate the rice actin and maize ubiquitin gene promoters, respectively (Fig. 1*B*).

Generation of pVS11 Transformants. Plasmid pVS11 was delivered into wheat immature embryos through particle bombardment, and cultures were selected on bialaphos. From two transformation experiments, putative transgenic plants were regenerated and their T1 progenies were germinated on selection plates. From the first experiment of *ca.* 900 immature embryos, 11 putative transgenic lines were regenerated, of which only 2 lines, VS10.1 and VS10.2, yielded resistant T1 seedlings. From the second experiment of more than 1,200 embryos, 25 putative transgenic plants were regenerated, of which 2 lines, I-3 and I-5, yielded resistant T1 seedlings. With each of the four lines, a 3:1 segregation for bialaphos resistance was observed to indicate a single integration locus. The presence of *bar* in the four lines was confirmed by Southern hybridization.

Generation of *cre*-Expressing Lines. The simplest method to introduce Cre recombinase into the system is to cross in a *cre*-expressing locus. The *cre*-expressing line was obtained by particle bombardment of *ca.* 1,200 embryos with pP2-cre and pP2-bar DNA. Both constructs use the maize ubiquitin promoter (*P2*) to drive expression of *cre* or *bar* (Fig. 1*A*). From the screening of 15 putative transformants, 4 transmitted the resistance trait to progeny. Embryos of these four lines were assayed for Cre activity through bombardment with pHK52, which contains promoter *P2* transcribing an antisense *gus*-coding sequence flanked by oppositely oriented *loxP* sites.

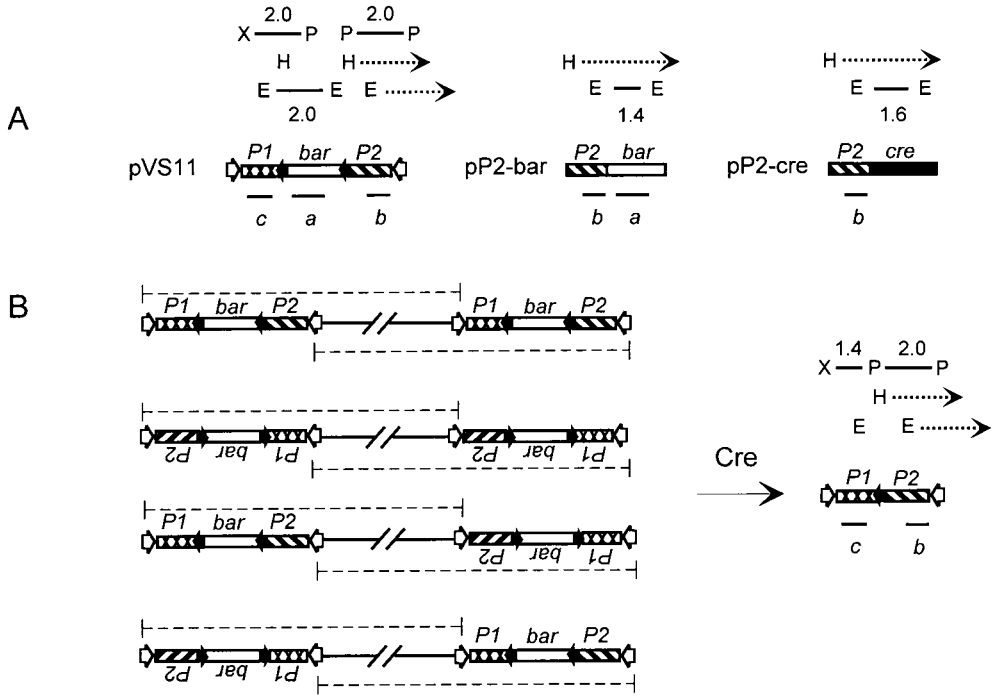

FIG. 1. (*A*) Schematic map (not to scale) of transformation vectors pVS11, pP2-bar, and pP2-cre. Only relevant features and DNA segments are shown. Solid arrowhead, *loxP*; open arrowhead, *lox511*. *P1*, rice actin promoter; *P2*, maize ubiquitin promoter; *bar*, phosphinothricin acetyl transferase coding region; *cre*, Cre recombinase coding region; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; X, *Xho*I. Restriction fragments that hybridize to the probes (*a*, *b*, or *c*) are in kilobases. Transgene–host DNA junction fragments that hybridize to probe *b* are indicated by dotted arrows. Not shown are the pUC18 vector backbones and the *nos3′* (transcription terminator) fragments that lie immediately downstream of *bar* and *cre.* (*B*) Resolution product from recombination of *lox* sites. pVS11 may integrate in multiple copies, with the outermost copies forming four possible configurations. Recombination between the outermost *lox511* sites (indicated by dashed lines) resolves the complex locus into a unit copy of pVS11. Recombination between *loxP* sites removes the selectable marker.

Cre-mediated recombination is expected to invert the *gus* fragment and permit expression of sense-strand mRNA. The T1 embryos of lines Cre34 and Cre37 stained blue after bombardment with pHK52, with Cre37 as the stronger-expressing line. The presence of *bar* and *cre* DNA in Cre37 was confirmed by Southern hybridization. Fortuitously, subsequent analysis of T2 progenies showed that Cre37 contains segregating *bar* and *cre* loci, and a Cre37 segregant (Cre37S) was identified that contains *cre* but lacks the *bar* locus. Initially, Cre37 and, subsequently, Cre37S were used for crosses with pVS11 transformants.

Marker Excision. T2 plants of each of the pVS11 lines were crossed with either Cre37 or Cre37S (Table 1). Except for Cre37S, the *bar* marker is present in all of the parental lines. Genomic DNA of the parents and their F1 progenies were cleaved with *Eco*RI and hybridized to the *bar*-coding region (Fig. 1, probe *a*). The VS10.1, VS10.2, I-3, and I-5 parents all have an internal, 2-kb *Eco*RI band corresponding to *P1-bar* (Fig. 2). In representative F1 plants derived from crosses to Cre37 (for VS10.1 and VS10.2) or Cre37S (for I-3 and I-5), this 2-kb *P1-bar* band is no longer found. This is consistent with Cre-*loxP*-mediated excision of the *bar* gene. Cre37 produces an internal, 1.4-kb *Eco*RI fragment corresponding to *P2-bar*, and this same band was observed in the F1 progenies derived from crosses with VS10.1 and VS10.2.

The excision of *bar* should fuse *P1* and *P2* linked by *loxP*. Using primers corresponding to *P1* and *P2*, a 0.8-kb fragment corresponding to the new *P1-loxP-P2* junction was detected (Fig. 2). In contrast, a band of 1.8 kb representing the *P1-loxP-bar-loxP-P2* linkage was amplified from VS10.1, VS10.2, I-3, and I-5, but not from the F1 progenies. A PCR product was not detected from Cre37, Cre37S, or nontransformed plants. The *loxP* sequence of the 0.8-kb PCR product is functional, because it could recombine with a plasmid substrate *in vitro* (data not shown). The nucleotide sequence of a representative PCR fragment confirmed that a precise *P1-loxP-P2* junction was formed (data not shown).

Reduction of Copy Number and Segregation of the *cre* Locus. To identify transgene–host DNA junctions, genomic DNA of parental and progeny lines were probed with a subfragment of the *P2* sequence (Fig. 1, probe *b*). With VS10.1, VS10.2, and I-3, *Hin*dIII-cleaved DNA showed intense hybridization to fragments in the 5- to 6-kb range (Fig. 3 *A–C*). This hybridization is likely to represent multiple copies (in direct and/or inverted orientation) of pVS11, because they are not found in the F1 progenies. The F1 lanes show only bands that could be attributed to the Cre37 or Cre37S genome or to a likely transgene–host DNA junction.

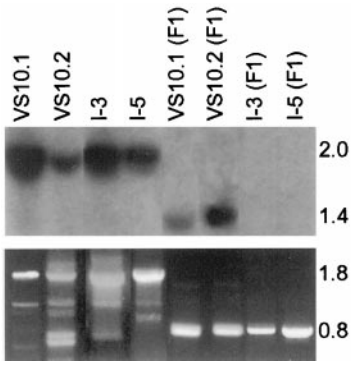

FIG. 2. Excision of *bar* gene in F1 progenies. (*Upper*) *Eco*RI-cleaved DNA of VS10.1, VS10.2, I-3, I-5, and their representative F1 outcross (to Cre37 or Cre37S) progenies were hybridized to *bar* (probe *a*, Fig. 1). (*Lower*) PCR using primers corresponding to *P1* and *P2* sequences. Parental DNA shows a 1.8-kb band resulting from the presence of *bar* between *P1* and *P2*. Representative F1 progenies show a 0.8-kb band from the formation of a new *P1-loxP-P2* junction.

Fig. 3 shows representative F2 progenies, derived from self-fertilized F1 plants, which have segregated away the *cre* locus. In each line, only a single, putative transgene–host DNA junction is found (Fig. 3, arrowheads). For the I-5 line, *Hin*dIII-cleaved DNA showed hybridization to high-molecular-weight DNA that migrated at limiting mobility. However, *Eco*RI-cleaved DNA is well resolved and shows hybridization to two bands of ≈2.5 and ≈3.5 kb (Fig. 3*D*). If both bands represent transgene–host DNA junctions, this would imply that the two molecules integrated as tandem, inverted copies or in whichever orientation but interspersed by host DNA. In the F1 progeny, a ≈1.6-kb, Cre37S-specific band

Table 1. Genotype of F2 segregants that harbor pVS11 DNA

Parents			F2 progeny				
Female donor	Male donor	F1 line	No. analyzed	pVS11 copy number	Presence of *cre*	Presence of *P2-bar*	Presence of amp
VS10.1	Cre37	A	4*	1	−	−	−
			7	1	−	+	+
			9	1	+	ND	ND
Cre37	VS10.1	B	13	1	−	+	+
			3	1	+	ND	ND
			4†	M	+	+	+
VS10.2	Cre37	C	2*	1	−	−	−
			1	1	−	+	+
			3	1	+	ND	ND
			4	M	+	+	+
Cre37	VS10.2	D	2	1	−	+	+
			6	1	+	ND	ND
I-3	Cre37S	E	1	1	−	−	+
			6	1	+	−	+
			2	M	+	−	ND
I-5	Cre37S	F	1	1	−	−	+
			4	1	+	−	+

F2 progenies are derived from the self-fertilization of a representative F1 plant found to have resolved the multicopy locus of the parent. F2 segregants listed do not include those that segregated away the pVS11 locus. M, multiple copies; ND, not determined.

*F3 progeny obtained from outcross to wild type showed the same genotype.

†Circular *loxP-bar* fragment detected.

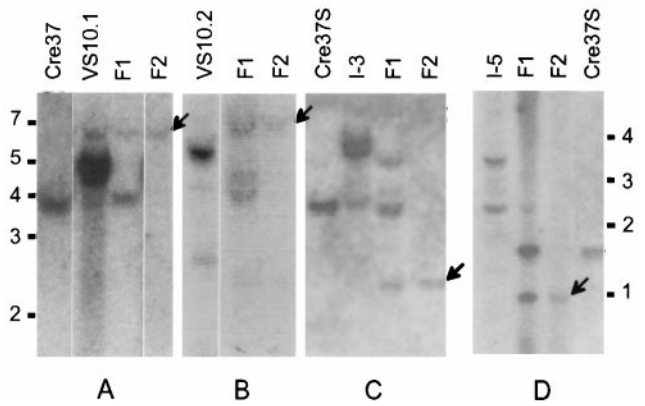

FIG. 3. Reduction of copy number and segregation of *cre* locus. Genomic DNA of parental lines and their F1 and F2 progenies were cleaved with *Hin*dIII (*A*, *B*, and *C*) or *Eco*RI (*D*) and hybridized with *P2* (probe *b*, Fig. 1).

is found, along with a faint, ≈2.5-kb band and a new, ≈1-kb band. Upon segregation of the *cre* locus, only this ≈1-kb band remained in the F2 progeny.

Possible Inversion of the Transgene. Because the resolved, single-copy transgene is flanked by *lox511* sites of opposite orientation (Fig. 1*B*), Cre-mediated recombination between the two sites would invert the transgene. If an inversion were to take place, the *P2* hybridization probe would detect a new *P2*–host DNA junction. This was the case with lines I-3 and I-5. Each F1 plant showed both the original and a new *P2*–host DNA junction that was not present in its parent. This indicates that the F1 plant was chimeric for the inversion and harbored both transgene orientations. By the next generation, the representative F2 plant chosen for analysis showed only the new *P2*–host DNA junction. In contrast, transmission of a transgene inversion was not observed for lines VS10.1 and VS10.2. Each F2 plant revealed a *P2*–host DNA junction fragment of the same size as that detected in the primary transformant.

Target Site Integrity. F2 or F3 plants that have segregated away the *cre* locus were analyzed for the expected structure. When cleaved with *Pst*I and probed with *P2* DNA (probe *b*), a 2-kb *P2* fragment was observed (Fig. 1*B* and Fig. 4), consistent with the size in the primary transformants. When cleaved with *Xho*I and *Pst*I and probed with *P1* DNA (Fig. 1*B*, probe *c*), a 1.4-kb band representing the full-length *P1* fragment was found. In contrast, the parents showed a 2-kb fragment because of the *Pst*I site within the downstream *bar* gene (Fig. 1*A* and Fig. 4). The data indicate that both promoters in the progeny plants are intact and are consistent with the intended structure depicted in Fig. 1*B*.

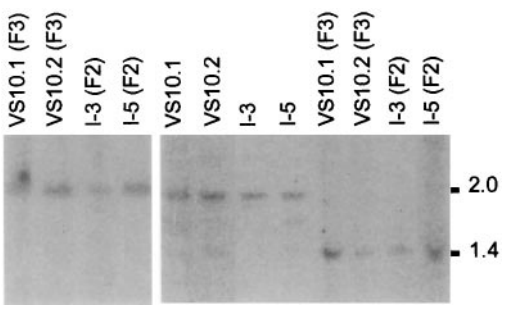

FIG. 4. Structure of the resolution product. Genomic DNA of F3 or F2 plants derived from each of the parents were cleaved with *Pst*I (*Left*) and hybridized to P2 (probe *b*, Fig. 1) or a combination of *Pst*I and *Xho*I (*Right*) and hybridized with *P1* (probe *c*, Fig. 1).

The presence of vector DNA was indicated by hybridization to the plasmid-encoded *amp* gene. Hybridization signals indeed were found in the *cre*- and *bar*-deficient F2 segregants derived from I-3 and I-5 (Table 1). Interestingly, of the six F3 segregants derived from VS10.1 and VS10.2 that had segregated away the *cre* and *bar* markers (Table 1, F1 lines A and C), none showed hybridization to *amp*. This could have arisen if all copies of the *amp* gene were internal to the outermost *lox* sites and, therefore, were deleted by the Cre-*lox* reaction.

Chimerism in the Germ Line. Table 1 summarizes the molecular analysis of F2 segregants derived from six F1 lines that showed resolution of the integration locus. It was surprising to find that 20–40% of the F2 progenies from three lines (Table 1, lines B, C, and E) had maintained multiple copies of the transforming DNA. Thus, although Southern analysis of the F1 plants indicated complete resolution of the integration site, at least when leaf tissues were analyzed, germ-line cells in these three lines must have been chimeric. Furthermore, these F2 progenies show the presence of *cre* DNA. Whether *cre* is active in these plants has not been determined.

Rare Instances of an Unexpected Pattern. The F1 plant derived from the Cre37 ♀ × VS10.1 ♂ cross (Table 1, line B) led to the molecular examination of 20 F2 progenies, of which 4 maintained multiple copies of the transforming DNA. More interestingly, an unexpected band was found when hybridized to *bar* DNA. The probe detected three *Eco*RI bands of sizes 1.0, 1.4, and 2.0 kb (Fig. 5, lane 2). The 1.4- and 2.0-kb fragments correspond, respectively, to *P2-bar* from the Cre37 genome and *P1-bar* of the unresolved pVS11 integration locus. Incomplete excision is consistent with the detection of multiple copies of pVS11 DNA. When *Hin*dIII-cleaved DNA was probed with *P2* DNA, the 5-kb fragment characteristic of the unresolved locus was observed. The presence of the 1.0-kb *bar* band, however, was unexpected. Further analysis suggested that it is a circular *loxP-bar* molecule (with *nos*3′; see Fig. 1 legend). Its presence and transmission coincided with the presence of both the unresolved integration locus and *P2-cre*. Further work is needed to determine why this excision product is maintained whereas most others are lost.

Concluding Remarks. Single-copy transgenic lines are desirable for a variety of reasons. It permits speedier structural and functional characterization, simpler structural documentation, and potentially greater stability in gene structure and expression. The current approach of screening for single-copy insertions by DNA hybridization is capital- and labor-intensive. It is also unpredictable, because success is highly dependent on obtaining a large number of transformants. With the strategy described in this work, the resolution of a complex integration locus through site-specific recombination is expected to produce a unit-copy transgenic plant from virtually 100% of the transformants. This will be of greatest value for plants from which transformants are relatively difficult to obtain. Such is the case with wheat, and with the commercial lines of otherwise transformable plant species, where DNA transformation is often far less efficient than with laboratory cultivars. The direct introduction of DNA into commercial

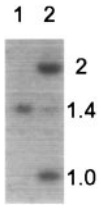

FIG. 5. Rare instance of unexpected pattern. Genomic DNA of F2 plants was cleaved with *Eco*RI and hybridized with *bar* (probe *a*, Fig. 1). A majority of F2 plants shows only the 1.4-kb band derived from pP2-bar of the *cre* genome (lanes 1), but a few individuals show additional *bar* hybridization bands (lane 2).

lines would save generations of crosses needed for introgression of the desired trait (39).

Transgene inactivation is affected by several genomic and environmental factors (reviewed in ref. 3). There may be one or several cellular mechanisms triggered by these factors to cause transgene silencing (13, 40). To stabilize gene expression, it may be necessary to control the structure and location of the integration locus in addition to appropriate promoter activity (8, 13). This calls for greater precision in the introduction of DNA molecules. Homologous recombination would be ideal but currently is not a practical option (41). Recombinase-mediated, site-specific integration, however, has shown promise in model plant systems (34, 42). The marker-free resolved sites generated in the present study would be ideal for site-specific insertion of transgenes into the wheat genome.

An important consideration with the current strategy is the frequency of gene copies interspersed with host DNA, as opposed to being in a contiguous, tandem array. Two recent articles have described nontransgenic DNA interspersed among transgene copies in plants (14, 43). However, the origin of the nontransgenic DNA is not known. In mammalian gene-targeting experiments, a process known as "ectopic gene targeting" has been described (44). This is the situation in which the introduced DNA strand invades into the homologous locus and replicates a segment of DNA from that locus. The transgene linked with the copy of host DNA then integrates at a different site. If interspersed DNA were to be found and were derived from elsewhere in the genome, its removal along with the extra transgene copies most likely would be inconsequential. On the other hand, if the nontransgenic DNA were native to the locus, the resolution process would generate a deletion in the chromosome. In light of the latter possibility, for the practical implementation of this strategy, it would be prudent to obtain single-copy transgenic lines from several different progenitor lines.

The pregenome era has been a time in which gene discovery is the limiting factor for crop improvement through genetic engineering. With fewer genes to work with, substantial resources can be devoted to the engineering of fewer traits. For the upcoming postgenome era, however, an exponential increase in the number of genes will be uncovered through genome-sequencing efforts. The functions of a large set of genes will not be known. However, genetic transformation will remain both as a tool for defining gene function and a means for testing the commercial utility of new sequences. With a far greater number of genes to work with, the rate of crop improvement and commercialization of new traits will be much more dependent on the speed and efficiency in testing new DNA sequences in crop genomes. The replacement of conventional screening efforts for single-copy insertions with a resolution-based strategy would be a cost-saving step for the large-scale transformation of new sequences.

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