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Transgenic DNA integrated into the oat genome is frequently interspersed by host DNA

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ABSTRACT Integration of transgenic DNA into the plant genome was investigated in 13 transgenic oat (*Avena sativa* L.) lines produced using microprojectile bombardment with one or two cotransformed plasmids. In all transformation events, the transgenic DNA integrated into the plant genome consisted of intact transgene copies that were accompanied by multiple, rearranged, and/or truncated transgene fragments. All fragments of transgenic DNA cosegregated, indicating that they were integrated at single gene loci. Analysis of the structure of the transgenic loci indicated that the transgenic DNA was interspersed by the host genomic DNA. The number of insertions of transgenic DNA within the transgene loci varied from 2 to 12 among the 13 lines. Restriction endonucleases that do not cleave the introduced plasmids produced restriction fragments ranging from 3.6 to about 60 kb in length hybridizing to a probe comprising the introduced plasmids. Although the size of the interspersing host DNA within the transgene locus is unknown, the sizes of the transgene-hybridizing restriction fragments indicated that the entire transgene locus must be at least from 35–280 kb. The observation that all transgenic lines analyzed exhibited genomic interspersion of multiple clustered transgenes suggests a predominating integration mechanism. We propose that transgene integration at multiple clustered DNA replication forks could account for the observed interspersion of transgenic DNA with host genomic DNA within transgenic loci.

Plant transformation by microprojectile bombardment results in transgene integration patterns that generally exhibit multiple transgene copies and extensive rearrangements of the introduced DNA (for review see ref. 1). The mechanisms involved in genomic integration of transgenic DNA delivered by microprojectile bombardment are not well understood but can probably be considered in two stages. The first stage likely includes preintegration rearrangements in the introduced DNA as it is delivered to the nucleus, whereas the second stage involves the process of integration into the host genome. Plasmid DNA, usually used in microprojectile bombardment experiments in plants, may be subjected to mechanical shearing during particle preparation and bombardment processes, and may also be degraded by host-cell nuclease activity. In addition, nuclease activity may "nibble" the ends of linear DNA molecules (2). Ligation of these introduced DNA fragments into arrays of transgenic DNA is thought to precede genomic integration (3).

Very little is known about the process of integration of transgenes following microprojectile bombardment or other direct DNA delivery methods despite widespread use of these methods. The transgene integration process is difficult to monitor while it is occurring because of the low frequency of stable transformation events; the study of transgene integration patterns in stable transformants may allow elucidation of the transgene integration process. Direct DNA delivery usually results in transgenes integrated at one genomic locus (4–12) that consists of multiple, intact, and rearranged transgene copies (refs. 12–19; for review see ref. 1). Studies of DNA integration in mouse cells suggest that the transgenic DNA fragments delivered into the cell are engaged in end-to-end ligation before they are integrated into the genome, forming multiple transgene concatamers (20, 21). In plant transformation experiments, head-to-head or head-to-tail concatenation of the introduced transgenes also have been reported (2, 22–24), supporting the concept that contiguous arrays of transgenic DNA are integrated into a single genomic site. However, in a recent report (25), it appears that transgene loci in rice plants transformed by microprojectile bombardment may have host DNA separating closely linked transgene sequences. We analyzed the patterns of transgene integration in 13 transgenic oat lines produced by microprojectile bombardment that exhibited multiple intact and rearranged transgene copies integrated at single genomic loci. Within the transgenic loci in all of the lines, transgenic DNA fragments were interspersed with host genomic DNA, suggesting a predominant mechanism of transgene integration. Transgene integration at active DNA replication forks is proposed to account for the interspersion of transgenic DNA with host genomic DNA.

MATERIALS AND METHODS

Plant Transformation. Plasmids used in plant transformation are presented in Fig. 1. The plasmid pBARGUS (14) combines the selectable marker *bar* gene from *Streptomyces hygroscopicus* (26) and the reporter *gusA* gene from *Escherichia coli* encoding GUS (27) under the control of plant promoters. Some tissue cultures were cotransformed, in addition to pBARGUS, with one of four other plasmids, pH24, pMAV, pPAV, or pRPV. pH24 carried the Cauliflower Mosaic Virus (CaMV) 35S promoter/*Adh1* intron/*nptII* construct (28); the latter three plasmids contained the coat-protein genes of different strains of Barley Yellow Dwarf Virus (BYDV) under the control of the 35S promoter (29) (Fig. 1). For microprojectile bombardment, plasmid DNA was extracted following standard protocols and purified by equilibrium centrifugation in CsCl-ethidium bromide (30). A sample of the prepared plasmid DNA was subjected to electrophoresis in an agarose gel to verify that it was free from contamination. Plasmid DNA was coated onto tungsten particles and introduced by using microprojectile bombardment into embryogenic oat callus (31) initiated from immature embryos of the genotype GAF-30/Park (32). Phosphinothricin-resistant callus appeared 7–8 weeks after bombardment. Transgenic plants were regenerated on oat regeneration medium (33) containing

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Abbreviations: BYDV, barley yellow dwarf virus; GUS, β-glucuronidase; PFGE, pulse-field gel electrophoresis.

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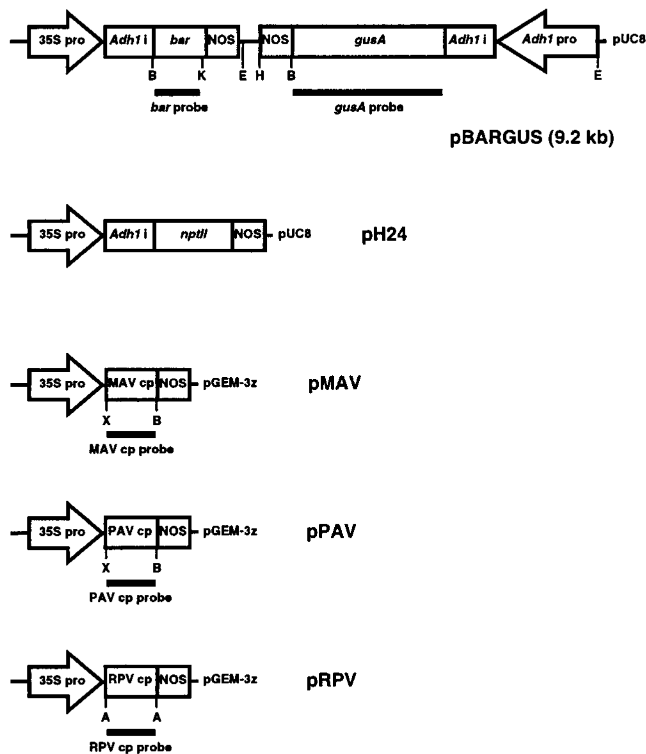

FIG. 1. Restriction maps of plasmids used for oat transformation. Positions of probes used in Southern blot analyses are indicated by black bars underneath the appropriate sequences. Not drawn to scale. 35S pro, Cauliflower Mosaic Virus (CaMV) 35S promoter; *Adh1* pro, promoter of the maize *Adh1* gene;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

3 mg/liter phosphinothricin. After 4–6 weeks shoots were transferred onto a hormone-free MS medium with phosphinothricin (31). Rooted plants were planted in potting soil mix and grown to maturity in controlled-environment growth chambers (34).

Southern Blot Analyses. DNA from transgenic tissue cultures was extracted as described by Saghai-Maroof *et al.* (35) and from plant leaf tissue as described by Hu and Quiros (36). DNA for pulsed-field gel electrophoresis (PFGE) was extracted from plant leaf tissue as follows. Five grams of fresh tissue was ground in liquid nitrogen. After the nitrogen had evaporated, 10 ml of nuclei isolation buffer (37) supplemented with 2% sarkosyl was added to the powder and mixed. The homogenate was extracted twice with phenol/chloroform 1:1 (vol/vol), and the DNA was precipitated with 2 ml of 3 M sodium acetate and 30 ml of 95% ethanol. The DNA was resuspended in 2 ml of TE (10 mM Tris, pH 7.6/1 mM EDTA, pH 8.0), purified twice with Nucleon Phytopure silica resin (Nucleon Biosciences, Coatbridge, U.K.) and precipitated with 200 μl of 8 M ammonium acetate and 5 ml of 95% ethanol.

About 10 to 30 μg of DNA was digested overnight with restriction endonucleases. The DNA digests were separated in 1.0% agarose gels and 1× TAE buffer (40 mM Tris·acetate/1 mM EDTA, pH 8.0). PFGE was performed by using a CHEF (Clamped Homogeneous Electrical Field) apparatus (Bio-Rad) in 0.8% agarose gels and 1× TAE buffer. The PFGE gels were run at 1.0 V cm^{-1} with a switch time of 2–15 sec ramped over 72 hr. DNA was transferred from agarose gels onto Immobilon N (Millipore) or Hybond N+ (Amersham) membrane according to manufacturer's recommendations.

Double-stranded DNA probes were ^{32}P-labeled with the Rediprime (Amersham) or the Prime-a-gene (Promega) kit. To detect transgene coding regions, a 1.8-kb *Bam*HI/*Sst*I fragment from pBI221 containing *gusA* (27) and a 536-bp *Bam*HI/*Kpn*I fragment from pBARGUS containing *bar* (26) were used as probes. Coding regions of the BYDV coat-protein genes (29) were used to detect the presence of the cotransformed plasmids (Fig. 1). A mixed probe, used for detection of the transgene integration sites, consisted of DNA of the five plasmids used in plant transformation (pBARGUS, pH24, pMAV, pPAV, and pRPV) labeled separately with ^{32}P and combined for hybridization. Membranes were analyzed by autoradiography with the X-Omat AR5 film (Eastman Kodak).

RESULTS

Genomic Integration and Arrangement of Transgenic DNA. Transgenic oat lines were produced using microprojectile bombardment of 12- to 24-week-old embryogenic callus initiated from the oat genotype GAF-30/Park (31). Plants regenerated from a single transgenic tissue culture (generation T_0), as well as self-pollinated progeny of the regenerated plants, were designated as a "transgenic line." Thus, each transgenic line traced back to a different tissue culture and represented an independent transformation event. Thirteen transgenic lines that exhibited single transgene loci were selected for further characterization. Lines 300 and 301 were transformed only with the plasmid pBARGUS. All other lines were produced by cotransformation of pBARGUS with one of four other plasmids, pH24, pMAV, pPAV, or pRPV (Fig. 1). The *bar* and *gusA* probes from pBARGUS (Fig. 1) hybridized to high-molecular-weight DNA in undigested DNA samples from the transgenic plant lines, indicating that the transgenes were integrated into the plant genome (data not shown). The DNA from the transgenic plants also was digested with *Eco*RI and *Bam*HI, which release a 0.8-kb restriction fragment containing the *bar* coding region and a 3.6 kb-fragment containing the *gusA* coding region (Fig. 1). Restriction patterns of transgenic DNA were complex in most of the lines (Fig. 2*A*). In addition to the restriction fragments of the expected size, fragments larger and smaller than the expected size were detected that represented rearrangements of the transgene DNA. There were no rearranged fragments of the same size recurrently observed in different lines (Fig. 2 *A* and *B*) indicating that all transformation events were unique and the rearrangements of transgenic DNA occurred randomly.

DNA from transgenic plants also was digested with *Hin*dIII, which has only one restriction site in the version of pBARGUS that we used and was expected to yield fragments corresponding in size to the repeats in a pBARGUS concatamer if plasmid concatenation had occurred. Multiple different-sized *Hin*dIII restriction fragments hybridizing to the *gusA* probe with roughly equal intensity (Fig. 2*B*) indicated that concatenation of full-length plasmid copies was not the predominant mode of transgene organization in the plant genome.

A transgene copy number reconstruction analysis was performed on the 13 transgenic lines (data not shown). Several plants from each line were analyzed to minimize errors caused by unequal loading of DNA. In 11 of the lines, the number of *bar* and *gusA* copies varied from 1 to 5 copies per line (with an average of 2.6 copies of *bar* and 3.1 copies of *gusA* in these lines). The exceptions were lines 504 and 903, which had approximately 10 and 7 copies of *bar* and 6 and 20 copies of *gusA*, respectively. The estimated number of *bar* copies usually corresponded to the number of *gusA* copies, although in some lines the copy number of *bar* and *gusA* were very different, suggesting that some integrated plasmid DNA fragments contained one of the transgenes but lacked the other.

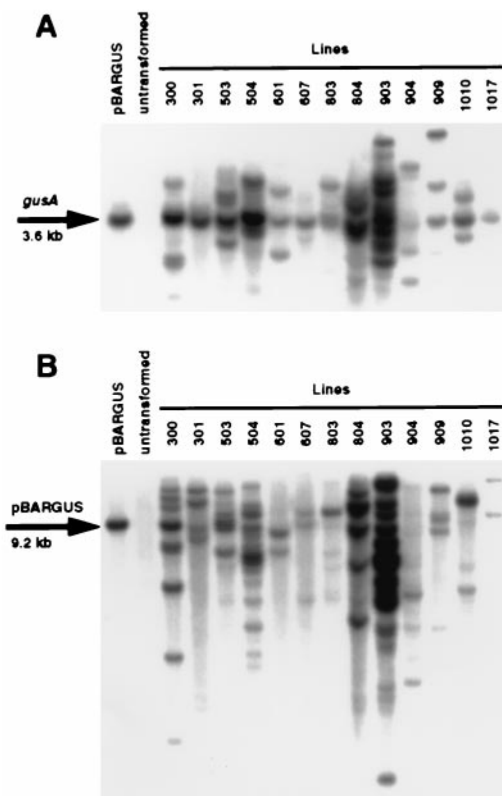

FIG. 2. Transgene integration patterns in transgenic oat lines. DNA was restricted with *Bam*HI and *Eco*RI (*A*) or *Hin*dIII (*B*) and probed with *gusA*. *Bam*HI and *Eco*RI release from pBARGUS a 3.6-kb fragment containing the *gusA* coding region (see Fig. 1). *Hin*dIII has a single restriction site on pBARGUS.

Transgenic DNA Behaves as a Single Genetic Locus. To characterize the arrangement of integrated transgenes in the plant genome, transgene segregation was determined in progeny of the 13 plant lines. Segregation of transgenic DNA in T_1 and/or T_2 generation progenies of transgenic plants was analyzed by probing Southern blots with coding sequences of *bar*, *gusA*, the BYDV coat-protein genes from the three cotransformed BYDV plasmids, and the entire pBARGUS sequence, which could also detect common sequences present on the cotransformed plasmids (Fig. 1). DNA from segregating progenies was digested with *Bam*HI and *Eco*RI, which released fragments containing the *bar* and *gusA* transgenes (Fig. 1). The analyses were repeated with *Hin*dIII, which has only a single restriction site on pBARGUS and produced multiple transgene restriction fragments of unique size as shown in Fig. 2*A*. In each line, all fragments of pBARGUS and the cotransformed plasmids cosegregated in the progeny, indicating that the transgenic DNA was integrated in single loci (Table 1; for example see Fig. 3). Moreover, in segregating progenies of the transgenic lines, the transgene integration patterns were identical to the patterns present in the progenitor tissue cultures, which further verified the transmission of all transgene fragments to the progeny of regenerated plants as a single locus. Analyses of segregation of the transgene phenotypes performed on a large number of individuals from the 13 single-locus lines also indicated the presence of one functional transgene locus (see ref. 38 for details).

Interspersion of Transgenes with Genomic DNA. The arrangement of the transgenic DNA within the integration loci in the 13 single-locus lines was characterized by Southern blot analyses performed with *Bst*EII, which does not cleave pBARGUS or any of the cointroduced plasmids. *Sca*I, which cleaves the cointroduced plasmids but not pBARGUS, was

Table 1. Numbers of plants exhibiting identical transgene integration patterns detected among segregating transgene-positive progeny within a line indicating the presence of single transgene loci

		No. of plants analyzed	
Line	Introduced plasmids	Transgene-positive plants	Transgene-negative plants
300	pBARGUS	26	4
301	pBARGUS	45	35
503	pBARGUS + pH24	6	2
504	pBARGUS + pH24	25	6
601	pBARGUS + pRPV*	25	8
607	pBARGUS + pMAV	13	16
803	pBARGUS + pMAV	13	2
804	pBARGUS + pPAV	14	7
903	pBARGUS + pRPV	5	10
904	pBARGUS + pPAV	4	5
909	pBARGUS + pRPV	3	2
1010	pBARGUS + pMAV*	4	2
1017	pBARGUS + pMAV*	3	2

Southern blots were probed with *bar*, *gusA*, the entire pBARGUS sequence, and the coding regions of the three BYDV coat protein genes. To detect unlinked transgene fragments with a probability greater than 0.95, 11 transgene-positive T_1 individuals or 7 transgene-positive T_2 individuals need to be analyzed (8 and 5, respectively, for $P > 0.90$). Alternatively, analysis of 3 transgene-negative T_1 or 4 transgene-negative T_2 plants is sufficient for $P > 0.95$ (2 and 3, respectively, for $P > 0.90$). T_1 and/or T_2 plants were sampled based on the presence or absence of transgene phenotypes. Thus, the ratio of transgene-positive to transgene-negative plants does not reflect the transgene segregation ratio in the line.

*BYDV coat protein gene was not detected in the transgenic plants nor in the progenitor tissue culture.

also used in the analyses of lines 300 and 301 that were transformed with only pBARGUS. After restriction digestion, DNA was separated by PFGE and analyzed by Southern blot analysis. Completeness of the restriction digestion was verified by adding lambda phage DNA to the plant genomic DNA before restriction digestion. After this procedure, the expected size-restriction fragments were detected when probing the Southern blots with total lambda DNA. Additional confirmation of complete sample digestion was obtained by probing Southern blots with an oat restriction fragment length polymorphism (RFLP) probe CDO 638 (39), which consistently detected the same restriction fragments (ranging from 6 to 23 kb in size) in all samples treated with the same restriction endonuclease (data not shown). All 13 single-locus lines exhibited multiple DNA fragments that hybridized to the mixed probe consisting of all transformed plasmids (Fig. 4). From 2 to more than 12 DNA fragments were observed, with

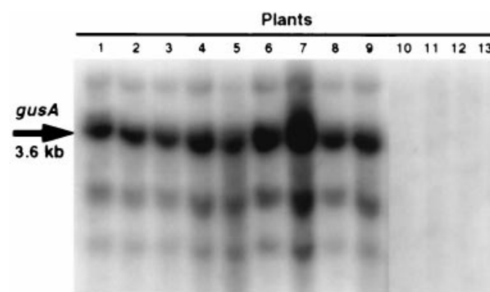

FIG. 3. Cosegregation of transgene restriction fragments in a sample of 13 T_2 progeny plants of line 300. DNA was restricted with *Bam*HI and *Eco*RI and probed with *gusA*. Plants in lanes 1–9 exhibit all transgene-hybridizing restriction fragments detected in this line. Plants in lanes 10–13 show no transgene-hybridizing restriction fragments.

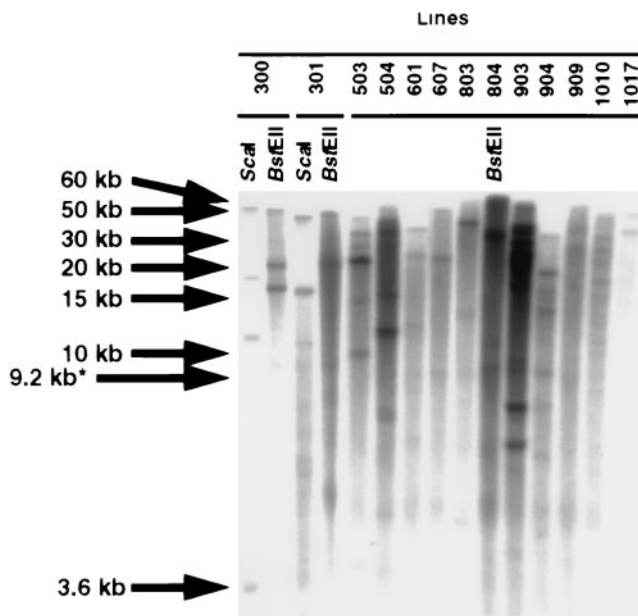

FIG. 4. Multiple transgene integration sites in single-locus transgenic oat lines. DNA was separated by PFGE, Southern blotted, and probed with a mixed probe containing all plasmids used for plant transformation (pBARGUS, pH24, pMAV, pPAV, and pRPV). The plasmid pBARGUS is 9.2 kb (*).

an average number of about 5.5 fragments per line. Two transgene-hybridizing fragments were present in lines 601, 803, and 1017, whereas more than two fragments were detected in all other lines (Fig. 4). The size of the fragments ranged from 3.6 to about 60 kb. Some fragments in lines 300 and 301 were smaller than pBARGUS (Fig. 4), which was the only plasmid used to transform these lines, indicating that these fragments contained truncated plasmid copies.

Summing the molecular sizes of transgene restriction fragments detected on the pulse-field gel allowed an estimation of a "minimum size" of the transgene integration loci. These estimates assumed that the transgene-containing restriction fragments detected by PFGE were arranged tandemly within a locus. The predicted "minimum sizes" of transgenic loci ranged from 35 to nearly 280 kb. This analysis did not allow size estimation of the intervening plant genomic DNA. However, in line 300, digestion with *Bst*EII produced three different restriction fragments, whereas *Sca*I digestion resulted in four restriction fragments (Fig. 4), indicating that one of the intervening host DNA fragments was smaller than 50 kb (the size of the largest *Bst*EII fragment).

DISCUSSION

The multiple restriction fragments in the 13 single-locus transgenic oat lines appeared to result from interspersion of transgenic DNA with host genomic DNA. The interspersing host DNA most likely contained sites for the restriction enzymes that did not digest the introduced plasmid DNA. Other mechanisms that could account for the presence of the *Bst*EII and *Sca*I restriction sites seem far less likely. For example, random creation of new restriction sites by plasmid rearrangements occurring during transgene integration probably could not produce the observed number of restriction sites (up to 11 per line). Furthermore, the plasmid DNA used for microprojectile bombardment was highly purified and no contamination by foreign DNA was detected in the plasmid preparations by using electrophoretic gel analysis.

Our results indicate that interspersion of transgenic DNA fragments with host DNA within the transgene locus is the predominant mode of organization of transgenic loci in oat containing multiple transgene copies. Recently, Kohli *et al.* (25) reported that 6 of 16 single-transgene-locus rice lines exhibited intervening sequences within the transgene loci detected by restriction enzymes that did not cleave the introduced plasmid DNA. In the rice lines in which the intervening sequences were observed, only a few interspersed transgene fragments were detected, whereas in oat up to 12 interspersed fragments were observed in the transgenic lines. The general features of transgene integration in transgenic oat and rice (25) such as a variable number of integrated transgene copies and complexity of the transgene restriction pattern with a high frequency of altered-size transgene copies are similar to the transgene integration patterns reported in most plant transformation experiments using microprojectile bombardment and other direct DNA delivery methods (refs. 4–19; see also ref. 1). This makes it likely that frequent transgene interspersion with the host genomic DNA that we observed in transgenic oat may also be frequent in other plant species and perhaps other eukaryotes.

Genomic integration of transgenes has been associated with DNA replication and break-repair processes (24, 40–42). Increases in the frequency of transgene integration have been observed after applying agents that cause breaks in DNA, such as x-ray and UV irradiation, or after transformation during the S phase of the cell cycle, when an increased number of breaks in replicating DNA is present naturally. Radiation-hypersensitive mutants of *Arabidopsis thaliana*, which are most likely deficient in DNA break-repair, are also deficient in genomic integration of T-DNA (43). Illegitimate recombination has been proposed as a mechanism involved in integration of transgenic DNA after direct DNA uptake into rice protoplasts (44). One of the models for illegitimate recombination involves switching templates during the DNA replication process (45, 46). Involvement of DNA replication also was invoked by Gheysen *et al.* (47) to explain creation of rearrangements at the T-DNA ends and in the genomic target sequence in the process of T-DNA integration, although the authors postulated that illegitimate recombination during the integration of T-DNA occurs by breakage and rejoining of the DNA (47).

DNA replication in eukaryotes is believed to proceed in discrete clusters of DNA replication forks (48–50). Observations in pea demonstrated the presence of approximately 4,200 replication clusters per nucleus with, on average, 18 replication forks in each cluster (49). More recent observations in *Xenopus* nuclei replicating *in vitro* indicated 100–300 replication sites evenly distributed throughout the entire nucleus (48). Each site was a tight cluster of at least 300–1,000 replication forks. We propose that integration of transgenic DNA at a cluster of active replication forks could account for the interspersion of the integrated transgenes with host DNA that was observed in transgenic oat. Because of a relatively small number of replication clusters operating in the nucleus at any one time, the integration of all transgenic DNA fragments introduced into a nucleus could take place at just one cluster of replication forks, resulting in all of the transgene integration sites being very closely linked. No direct evidence is so far available for the proposed, or any other, mechanism explaining genomic integration of transgenes after direct DNA transfer in plants. It is possible that the interspersions within the transgene locus detected in oat were created after transgene integration by means of a transgene amplification process as observed in long-term experiments with transgenic tissue culture lines of petunia (51) and mouse (52). However, the irregular and complex structure of transgenic loci in oat, along with an application of selective pressure for a period of time that was most likely too short to result in transgene amplification (53), would argue against a gene-amplification hypothesis. More detailed analyses of transformation events that produce com-

plex integration patterns will undoubtedly contribute to elucidating transgene integration mechanisms.

It is unknown whether and how clustering may affect stability of transgenic DNA and expression of the transgenes. In an *Agrobacterium*-transformed *Petunia* population, different patterns of cosuppression were observed depending on the organization of the integrated T-DNA in direct, inverted, or dispersed repeats (ref. 54; see also ref. 55), suggesting that organization of transgenic DNA after integration into the plant genome may be as important as the transgene copy number for triggering transgene silencing. The majority of transgenic oat lines characterized in this study exhibited transgene silencing and abnormal segregation of the transgenic phenotypes (38). Transgenic lines with a more complex structure of the transgenic locus were more likely to exhibit *bar* silencing than lines with fewer transgene integration sites within the locus (data not shown). However, no such correlation was observed for silencing of *gusA*. For both *bar* and *gusA*, the frequency of transgene silencing did not depend on the number of transgene copies themselves (38). Clustering of transgenic DNA could facilitate interaction between multiple identical transgene copies and contribute to transgene silencing because direct DNA–DNA interaction between multiple transgene copies has been implicated in triggering silencing (56). In this case, a method for minimizing clustering of multiple copies of the introduced DNA may be desirable to improve expression of transgenes in genetically engineered plants.

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1. Pawlowski, W. P. & Somers, D. A. (1996) *Mol. Biotechnol.* **6,** 17–30.
2. Riggs, C. D. & Bates, G. W. (1986) *Proc. Natl. Acad. Sci. USA* **43,** 5602–5606.
3. Bates, G. W., Carle, S. A. & Piastuch, W. C. (1990) *Plant Mol. Biol.* **14,** 899–908.
4. Christou, P., Swain, W. F., Yang, N. S. & McCabe, D. E. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 7500–7504.
5. Jongsma, M., Koorneef, M., Zabel, P. & Hille, J. (1987) *Plant Mol. Biol.* **8,** 383–394.
6. Mouras, A., Saul, M. W., Essad, S. & Potrykus, I. (1987) *Mol. Gen. Genet.* **207,** 204–209.
7. Nehra, N. S., Chibbar, R. N., Leung, N., Caswell, K., Mallard, C., Steinhauer, L., Baga, M. & Kartha, K. K. (1994) *Plant J.* **5,** 285–297.
8. Peng, J., Kononowicz, H. & Hodges, T. K. (1992) *Theor. Appl. Genet.* **83,** 855–863.
9. Peng, J., Wen, F., Lister, R. L. & Hodges, T. K. (1995) *Plant Mol. Biol.* **27,** 91–104.
10. Potrykus, I., Paszkowski, J., Saul, M. W., Petruska, J. & Shillito, R. D. (1985) *Mol. Gen. Genet.* **199,** 169–177.
11. Qu, R., De Kochko, A., Zhang, L., Marmey, P., Li, L., Tian, W., Zhang, S., Fauquet, C. M. & Beachy, R. N. (1996) *In Vitro Cell. Dev. Biol.* **32,** 233–240.
12. Spencer, T. M., O'Brien, J. V., Start, W. G., Adams, T. R., Gordon-Kamm, W. J. & Lemaux, P. G. (1992) *Plant Mol. Biol.* **18,** 201–210.
13. Cooley, J., Ford, T. & Christou, P. (1995) *Theor. Appl. Genet.* **90,** 97–104.
14. Fromm, M. E., Morrish, F., Armstrong, C., Williams, R., Thomas, J. & Klein, T. M. (1990) *Bio/Technology* **8,** 833–839.
15. Gordon-Kamm, W. J., Spencer, T. M., Mangano, M. L., Adams, T. R., Daines, R. J., Start, W. G., O'Brien, J. V., Chambers, S. A., Adams, W. R., Jr., Willetts, N. G., *et al.* (1990) *Plant Cell* **2,** 603–618.
16. McCabe, D. E., Swain, W. F., Martinell, B. J. & Christou, P. (1988) *Bio/Technology* **6,** 923–926.
17. Register, J. C., III, Peterson, D. J., Bell, P. J., Bullock, W. P., Evans, E. J., Frame, B., Greenland, A. J., Higgs, N. S., Jepson, I., Jiao, S., *et al.* (1994) *Plant Mol. Biol.* **25,** 951–961.
18. Srivastava, V., Vasil, V. & Vasil, I. K. (1996) *Theor. Appl. Genet.* **92,** 1031–1037.
19. Wan, Y. & Lemaux, P. G. (1994) *Plant Physiol.* **104,** 37–48.
20. Anderson, R. A., Krakauer, T. & Camerini-Otero, R. D. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 2748–2752.
21. Folger, K. R., Wong, E. A., Wahl, G. & Capecchi, M. R. (1982) *Mol. Cell. Biol.* **2,** 1372–1387.
22. Finer, J. J. & McMullen, M. D. (1990) *Plant Cell Rep.* **8,** 586–589.
23. Hayashimoto, A., Li, Z. & Murai, N. (1990) *Plant Physiol.* **93,** 857–863.
24. Kartzke, S., Saedler, H. & Meyer, P. (1990) *Plant Sci.* **67,** 63–72.
25. Kohli, A., Leech, M., Vain, P., Laurie, D. A. & Christou, P. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 7203–7208.
26. Thompson, C. J., Movva, N. R., Tizard, R., Crameri, R., Davies, J. E., Lauwereys, M. & Botterman, J. (1987) *EMBO J.* **6,** 2519–2523.
27. Jefferson, R. A. (1987) *Plant Mol. Biol. Rep.* **5,** 397–405.
28. Callis, J., Fromm, M. & Walbot, V. (1987) *Genes Dev.* **1,** 1183–1200.
29. McGrath, P. F., Vincent, J. R., Lei, C.-H., Pawlowski, W. P., Torbert, K. A., Gu, W., Kaeppler, H. F., Wan, Y., Lemaux, P. G., Rines, H. W., *et al.* (1997) *Eur. J. Plant Pathol.* **103,** 695–710.
30. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY), 2nd Ed.
31. Somers, D. A., Rines, H. W., Gu, W., Kaeppler, H. F. & Bushnell, W. R. (1992) *Bio/Technology* **10,** 1589–1594.
32. Rines, H. W. & Luke, H. H. (1985) *Theor. Appl. Genet.* **71,** 16–21.
33. Bregitzer, P., Somers, D. A. & Rines, H. W. (1989) *Crop Sci.* **29,** 798–803.
34. Torbert, K. A., Rines, H. W. & Somers, D. A. (1995) *Plant Cell Rep.* **14,** 635–640.
35. Saghai-Maroof, M. A., Soliman, K. M., Jorgensen, R. A. & Allard, R. W. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 8014–8018.
36. Hu, J. & Quiros, C. F. (1991) *Theor. Appl. Genet.* **81,** 221–226.
37. Liu, Y.-G. & Whittier, R. F. (1994) *Nucleic Acids Res.* **22,** 2168–2169.
38. Pawlowski, W. P., Torbert, K. A., Rines, H. W. & Somers, D. A. (1998) *Plant Mol. Biol.* **38,** 597–607.
39. O'Donoughue, L. S., Kianian, S. F., Rayapati, P. J., Penner, G. A., Sorrells, M. E., Tanksley, S. D., Phillips, R. L., Rines, H. W., Lee, M., Fedak, G., *et al.* (1995) *Genome* **38,** 368–380.
40. Benediktsson, I., Spampinato, C. P. & Schieder, O. (1995) *Euphytica* **85,** 53–61.
41. Gharti-Chhetri, G. B., Cherdshewasart, W., Dewulf, J., Paszkowski, J., Jacobs, M. & Negrutiu, I. (1990) *Plant Mol. Biol.* **14,** 687–696.
42. Kohler, F., Cardon, G., Pohlman, M., Gill, R. & Schieder, O. (1989) *Plant Mol. Biol.* **12,** 189–199.
43. Sonti, R. V., Chiurazzi, M., Davies, C. S., Harlow, G. R., Mount, D. W. & Singer, E. R. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 11786–11790.
44. Takano, M., Egawa, H., Ikeda, J.-E. & Wakasa, K. (1997) *Plant J.* **11,** 353–361.
45. d'Alençon, E., Petranovic, M., Michel, B., Noirot, P., Aucouturier, A., Uzest, M. & Ehrlich, S. D. (1994) *EMBO J.* **13,** 2725–2734.
46. Tang, R. S. (1994) *BioEssays* **16,** 785–788.
47. Gheysen, G., Villarroel, R. & Van Montagu, M. (1991) *Genes Dev.* **5,** 287–297.
48. Mills, A. D., Blow, J. J., White, J. G., Amos, W. B., Wilcock, D. & Laskey, R. A. (1989) *J. Cell Sci.* **94,** 471–477.
49. Van't Hof, J. (1980) *Exp. Cell Res.* **129,** 231–237.
50. Van't Hof, J. & Bjerknes, C. A. (1977) *Chromosoma* **64,** 287–294.
51. Jones, J. D., Weller, S. C. & Goldsbrough, P. B. (1994) *Plant Mol. Biol.* **24,** 505–514.
52. Huberman, M., Berg, P. E., Currcio, M. J., DiPietro, J., Henderson, A. S. & Anderson, W. F. (1984) *Exp. Cell Res.* **153,** 347–362.
53. Bellini, C., Giordani, C., Lupotto, E., Locatelli, F., Cuzzoni, E., Avogadro, E., Castiglione, S. & Sala, F. (1992) *Plant Sci.* **82,** 193–200.
54. Jorgensen, R. A., Cluster, P. D., English, J., Que, Q. & Napoli, C. (1996) *Plant Mol. Biol.* **31,** 957–973.
55. Cluster, P. D., O'Dell, M., Metzlaff, M. & Flavell, R. B. (1996) *Plant Mol. Biol.* **32,** 1197–1203.
56. Matzke, M. A. & Matzke, A. J. M. (1995) *Plant Physiol.* **107,** 679–685.