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Targeted transgene integration: The way ahead to stable transformation

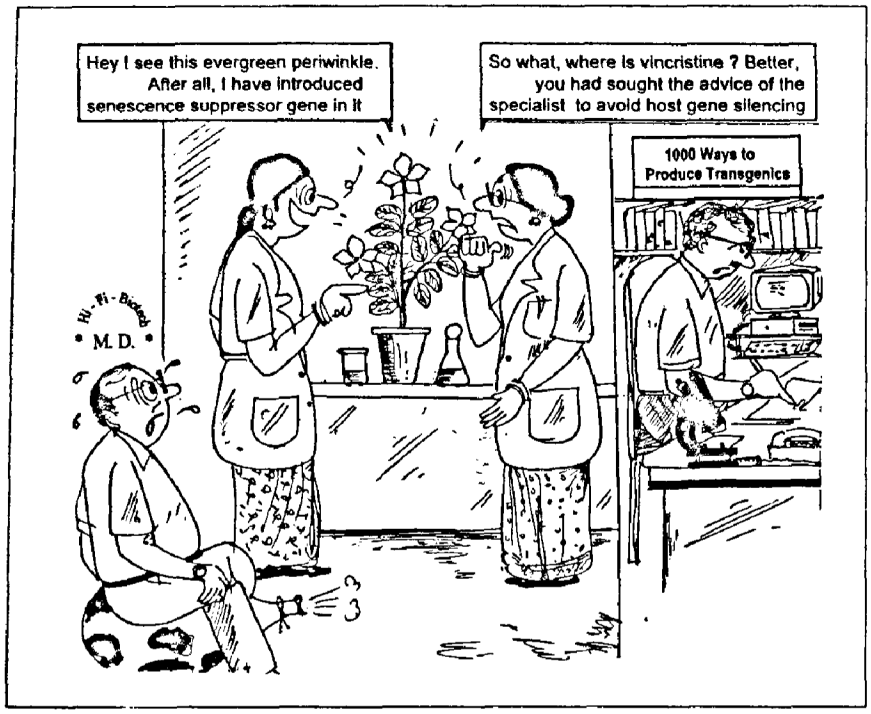
Stable integration and expression of introduced gene is essential to realize transgene advantage in the genetically-engineered crops. However, it has been clear for sometime that the introduced genes are not expressed uniformly in independent transgenic plants and that transgenic expression can change in successive generations. In addition, introduced genes can suppress the expression of related endogenous genes and/or transgenes already present in the genome[1]. The ability to recognize self, from non-self, a characteristic of the immune system, and the existence of self-DNA protection systems might modify the foreign DNA to make it nonfunctional or eliminate it altogether.

Although several factors contribute to inactivation and elimination of intrusive nucleic acids, methylation of intruder DNA and homology-dependent ectopic pairing are probably the major factors that contribute to transgene inactivation and gene silencing. The integration intermediates may be the prime targets for DNA methyltransferases that enforce repression of transgenes. Also, evidence is rapidly accumulating that silencing of single copy foreign genes, multicopy transgenes integrated either at the same locus or at unlinked loci frequently cause silencing of themselves and of homologous host sequences. The frequency of silencing encountered in multicopy transformants has led to the speculation that enhanced DNA : DNA pairing of the repetitive elements in such complex inserts might act as a signal for detection, resulting in highly efficient silencing. Therefore, suitable transformation constructs need to be designed to avoid host surveillance processes and facilitate predictable integration.

The preferred sites of transgene integration

The intrinsic ability of viral sequences or mobile genetic elements to excise and reinsert several times in search of a compatible genomic environment to attain stable integration is probably a mechanism for escaping the self-protection system of the host. The investigations into the sequence complexity of a genomic organization suggest that genomes are made up of 'isochores', i.e. very long stretches of DNA with high compositional homogeneity[2]. Carels *et al.*[3] found that almost all genes in maize are present in isochores covering an extremely low GC-range (1–2%) that represent only 10–20% of the genome. From this discovery, they developed the concept of 'gene space', in which genomic regions are represented by a single family of isochores[3], and noted that the gene space in maize corresponded to the only genome compartment in which certain mobile sequences can be transposed. The implication is that exogenous DNA arriving within the GC-rich gene space is likely to be actively transcribed, whereas DNA inserted into other regions is unlikely to be transcribed. Axiomatically, the GC-rich transgenes inserted into transcriptionally-active DNA remain functional, whereas AT-rich sequences in transgenes may mark them for inspection as invasive DNA. A precedent for this generalization has been observed in mammals: mobile sequences that have undergone numerous amplification and translocation events during the evolution of mammals, and several integrated viral sequences, were predominantly found in isochores of matching GC-composition[2]. Thus, it can be inferred that a matching genomic environment is essential for stable integration and expression of inserted sequences, and that excision and reinsertions may continue until the candidate transgene finds a compatible gene space in the host genome, else it would be eliminated/inactivated through the genome scanning mechanism[4]. The observations recorded from isochore studies[2,3] suggest that genomic systems exist that facilitate identification and inactivation (or elimination) of the foreign sequences that are compositionally different or incompatible from the genomic sites into which the insertions take place.

Iglesias *et al.*[5] have made an important discovery in relation to the stable integration and expression of transgene from the physical mapping of introduced DNA with respect to the spatial sites and sequence composition of chromosomal segments. Using fluorescence *in situ* hybridization to probe the physical location of transgene insertion, they demonstrated in tobacco that the stably-expressed inserts were present in the vicinity of telomeres, whereas the unstably-expressed inserts occupied intercalary and paracentromeric locations. Also, the stably-expressed loci comprised relatively simple T-DNA arrangements that were flanked on at least one side by plant DNA containing AT-rich regions that bind to nuclear matrices *in vitro*. It may be important to



mention here that the matrix attachment regions (MARs) that serve as a link between nuclear attachment sites and expressed sequence tags (ESTs), and facilitate gene expression, remain associated mainly with such telomeric regions[6].

Targeting transgene integration via homologous recombination

In plants, foreign DNA integrates randomly into the genome[7], regardless of whether a homologous region is included in the sequences. However, the controlled manipulation of genome by homologous recombination is of particular interest, because the product of the reaction can be determined in advance. Although homologous recombination predominates in transformation experiments using prokaryotes and lower eukaryotes, its frequency is low in higher eukaryotes on account of illegitimate recombination being the main pathway[8]. In spite of improved understanding of homologous recombination in plants[7], the goal of a feasible gene targeting achieved in lower eukaryotes seemed irreproducible in plants. However, it has now been reported for the moss *Physcomitrella patens*, that a predictable homologous integration is the predominant transformation pathway[9] and a natural gene in *Arabidopsis* could be knocked out[10]. The study by Schaefer and Zryd[9] marks a major breakthrough demonstrating that moss transformation frequencies increase tenfold when the transforming DNA contains sequences identical to the moss genome. Of course, there are still difficulties to check insertion of multiple copies, nevertheless, improvements in the transformation technique should ensure integration of only one copy of the homologous DNA per locus. Further, Schaefer and Zryd[9] speculate that there might be a close correlation between the haploid state and efficient homologous integration in eukaryotes, and therefore, it should be possible to make use of this information in higher eukaryotes where techniques for haploid cell transformation are available[11].

In view of the foregoing information, the following may be considered for efficient transgene integration:

1. Identification of isochores in the genome map of the target host is of value to design gene constructs amenable for stable integration. The isochore sizes and borders can be physically determined by compositional mapping, i.e. hybridizing probes for landmark genes or single copy anonymous sequences, both at the DNA and chromosome level.

2. Since MARs reduce variation in and increase transgene expression level, it is of utmost topographic value that the isochores that are predominant in subtelomeric regions may be targeted to serve as ideal sites for stable transgene integration.

3. The flanking regions of transgenes should preferably contain sequences homologous to compatible gene space of isochores so as to facilitate improved integration of transgenes by homologous recombination, and also attain a possible knock out of redundant endogenous sequences/allele replacement by double cross over.

4. The haploid cells in higher plants may possibly serve as a better experimental system to improve the efficiency of targeted transformation.

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