# Clean Vector Technology for Marker-free Transgenic Fruit Crops

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**Keywords:** gene transfer, excision, recombination, safe GMOs, apple, strawberry

## Abstract

**Marker-free transgenic crops confer several advantages over transgenic crops equipped with selection genes coding e.g. for antibiotic resistance. Firstly, the European Union has prepared a guidance document for risk assessment of GM-crops to be introduced in the environment (E.U. Joint Working Group on Novel Foods and GMO's, 2003). In this document based on compliance to consumer demands the EU encourages to "avoid or minimise the inclusion of superfluous transgenes or sequences". EU thus promotes the use of clean vector systems. Secondly, the number of selection genes allowing the preferential growth of transformed cells and tissues is limited. Often a gene transfer protocol for a specific crop or even a cultivar depends on the use of one specific selectable marker gene. Hence, stacking of genes within the same transgenic line is difficult once a selectable marker gene has been introduced. If these marker genes can be removed, the subsequent introduction of the next gene-of-interest is greatly facilitated. At Plant Research International a system has been developed for specific elimination of any introduced DNA/gene sequences using site-specific recombination combined with selection for successful removal using a negative selection system. Completely marker-free transgenic plants have been obtained using a model vector, both in an efficient transformation system (strawberry) as well as in a non-efficient transformation system (apple). Frequencies were more than adequate. Presently a versatile vector set providing a choice of several selectable markers and carrying a multiple cloning site for receiving cassettes of the gene-of-interest is available for application in, amongst others, fruit crops.**

## BACKGROUND

Genetically modified (GM) food products are looked upon with a certain degree of mistrust by the majority of European consumers. Almost 50% of consumers think that GM food is less safe for human consumption than non-GM food, 33% are not sure. Only 20% of the consumers know that there is no scientific basis to these doubts on food safety. While on the one hand players in the food production chain, such as breeding companies, growers/farmers and biotechnology firms, are convinced of the technical and economic benefits (Graff and Newcombe, 2003), the consumers on the other hand perceive risks to human health, to the environment and to biodiversity. In the USA and other countries around the globe, e.g. Argentina, Canada, China and India, GM crops are increasingly implemented in agriculture, however, the European input in research and development of GM crops has been reduced since 1998. This de-facto moratorium on the introduction of new GM crops has been in force since 1999. As a consequence of this, 51% of the European Small and Medium Enterprises (SMEs) at present do not use genetic engineering approaches, most of them indicated that there has been a change in their strategy in the last few years abandoning GM related R&D projects (Menrad and Menrad, 2003).

In July 2003, the ban on import of GM food products was lifted by the European Union (EU), with the proviso that all products containing more than 0.9% of EU-approved GM material have to be labeled. Also, the European Parliament has formulated guidelines based on their desire to provide European consumers with a choice to either accept or avoid the consumption of any GM or GM-derived food products. For this, completely separate production and processing chains will have to be set up. In practice,

XI$^{th}$ Eucarpia Symp. on Fruit Breed. & Genetics
Eds. F. Laurens and K. Evans
Acta Hort. 663, ISHS 2004

this might turn out to be too expensive for the industry, presenting yet another barrier to the implementation of GM food in Europe. Another consequence of this EU policy is the negative impact it has on imports of food from the USA and the reluctance of developing countries to grow GM crops out of fear of reducing their export possibilities to the EU.

In order to change the perception of EU consumers it might not be advisable to force GM crops and food upon them by enforcing official WTO rules and by pressure from the USA. Instead, consumers should regain confidence and trust in their scientists and government organizations. This can only be achieved by communicating clearly the benefits of particular GM crops and by highlighting the relevance of these crops to the individual consumer. Consumers should be able to relate to the goals of the modification and to the way these goals are achieved. If applicable, it should be explained that alternatives do not really exist or are less beneficial than the GM approach. In parallel to this, technical solutions to some of the concerns that consumers expressed are sought by science and can be applied.

In a 'Guidance Document for the risk assessment of GM plants and derived food and feed' by the Joint Working Group on Novel Foods and GMOs prepared for the EU, recommendations include the encouragement of notifiers to develop GM crops in which only DNA essential to the desired modification is introduced, e.g. clean vector technology. Overall, three principle ways are identified:

- avoid or minimize the inclusion of superfluous transgenes or sequences
- avoid or minimize superfluous expression of the transgene
- avoid or minimize the dispersal of transgenes in the environment.

Plant Research International has added to this list the preferential use of gene sequences or promoters which are species- or at least plant-derived. This, combined with PRIs own clean vector system and transparent communication on PRI arguments why and when to use GM technology, will hopefully contribute to a broader public acceptance of genetic modification of plants.

## CLEAN VECTOR TECHNOLOGY

Clean vector technology aims to produce GM plants with only the gene-of-interest as the newly introduced gene function, without any superfluous gene sequences. Primarily, the goal is to avoid the use or the continued presence of antibiotic resistance genes as selectable markers. Four approaches to achieve this can be followed.

### A. No Selectable Marker

Here, GM plants are produced by *Agrobacterium* inoculation followed by regeneration of shoots without the use of a selectable agent. This will lead to a (great) number of plants, the majority of which are non-transgenic. However, depending on the regeneration and gene transfer frequencies, some plantlets will be transgenic and they will have to be identified, e.g. by a dedicated PCR screening on DNA of several sets of pooled plants. A prerequisite is a regeneration/transformation protocol of high efficiency. So far, this method is limited to model species and a low number of specific crop cultivars, e.g. in potato.

### B. Cotransformation

In this system the selectable marker gene is physically separated from the gene-of-interest. This can be on different T-DNAs residing on the same or on separate binary vector(s). The separate binary vectors can be present in the same or in separate *Agrobacterium* strains. The two T-DNAs should become integrated in two genetically unlinked loci. After selection for the GM plants by growth on antibiotic or herbicide containing media subsequent segregation after sexual crossing of resistant regenerants should result in GM plants equipped only with the gene-of-interest. A prerequisite here is that the crop can be sexually propagated without losing too many traits or cultivar identity and this within a reasonable time frame. For vegetatively propagated crops or crops with a very long sexual cycle, such as tulip or apple, this approach is less feasible.

**C. Non-Antibiotic-Based Selection**

The most well known example in this respect is, of course, herbicide tolerance. However, there might be aspects of this that can be considered undesirable by certain consumer groups. An alternative system that was developed recently is based on metabolic processes, more particularly on the carbohydrate metabolism. The phospho mannose isomerase (PMI) system is based on the fact that plant cells in tissue culture cannot metabolize mannose and therefore, cannot grow on medium with this compound as a sole source of carbon. By providing GM cells with a gene converting mannose into fructose this barrier is overcome and GM plantlets can be obtained. As a consequence, however, they then still carry this bacterium-derived selectable marker gene at a stage where it is no longer actually needed, i.e. in crop cultivation.

**D. Excision by Recombination**

In this approach selectable marker genes or rather any unwanted (or no longer wanted) gene sequence, can be physically removed from the GM cells or regenerated GM plants. A recombinase enzyme working on two specific recombination sites is necessary. All of this has to be introduced into the primary transformants, next to the gene-of-interest and the selectable marker gene. Placing everything, which has to be removed ultimately, between the recombination sites ensures that in the final GM plant product only the gene-of-interest remains. Control over the recombinase activity is essential. This can be achieved by regulating or inducing expression of the recombinase gene or by inducing recombinase enzyme activity. Problems related to this technique are leakiness of the recombinase regulation, effectiveness of the induction process and recovery of 100% homogeneous recombined GM plants. Using a negative selection marker for transgenic, non-recombined cells or plantlets can cover this last aspect. This means that the prolonged presence of the negative selection gene will lead to cell death or an easily identifiable aberrant phenotype. Only the desired marker-free plants survive or will look normal.

The induced expression of a recombinase gene (Cre) is reported by Zuo et al. (2001), where a promoter is used comprising an estrogen receptor-based transactivator. Ebinuma et al. (1997) used the *ipt* gene as a phenotypical negative selection marker, the presence of which results in an aberrant phenotype. At Plant Research International, we use a synthetic copy of the recombinase (R) gene of *Zygosaccharomyces rouxii*, which is fused to the ligand binding domain (LBD) of a corticosteroid receptor. The R gene is continuously expressed, but the protein is inactive due to the translational fusion to LBD. Activity can be re-established by treatment with dexamethason (DEX). A dual positive-negative selectable marker consisting of a fusion between the *npt*II gene (positive) and the *cod*A gene (negative) are also a part of the PRI system. The *npt*II gene provides the cell with the competence to grow on medium supplemented with e.g. the antibiotic kanamycin, i.e. positive selection. The cytosine deaminase (*cod*A) gene product converts fluorocytosine (FC; non-toxic) into the toxic fluorouracil (FU), hence cells carrying this gene will die on medium with FC, i.e. negative selection. As stated earlier all of these elements are placed between the two Rs recombination sites.

The steps required for the production of marker-free GM plants are:

1) Inoculation of explants with *Agrobacterium* carrying the appropriate vectors as binary plasmids, according to established protocols.
2) Selection of transgenic material by growth on media containing antibiotics, again according to existing protocols
3) Induction of recombinase activity by applying a DEX treatment
4) Selection for marker-free plantlets by applying regeneration protocols in the presence of FC as a negative selectable agent for non-successful recombination events
5) A thorough molecular characterization of the end products should confirm the transgenic and marker-free nature of the plants produced.

The model construct has been successfully applied in potato, strawberry and apple (Schaart et al., 2004); the first two representing efficient transformation systems and the latter being much more recalcitrant. So far, the system of recombinase activity inhibition

and induction by DEX did not prove to be 100% effective. However, combined with the existing protocols and tissue culture/regeneration expertise in the crops tested, it easily led to many homogeneous marker-free GM plants, indicating that 100% effectiveness is not required.

At present, we are testing the efficiency of these new vectors based on the 'standard' vector. In apple, a gene conferring scab resistance derived from barley was equipped with the rubisco promoter of chrysanthemum for application in our marker-free system; in strawberry a strawberry disease-resistance gene was combined with a strawberry tissue-specific promoter. This concurs with the Plant Research International strategy for the production of a new generation of GM crops: marker-free, containing preferably a combination of a plant (or species) derived promoter and a plant (or species) derived gene (see earlier). This, we sincerely hope, will lead to a broader acceptance of these crops by a majority of the public, although it is acknowledged that this approach represents merely a technical solution to some of the objections, and not an answer to every issue raised by consumers at the moment.

Plant Research International is willing to enter into collaborations with interested parties to apply the system on specific crops of interest.

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## Figures

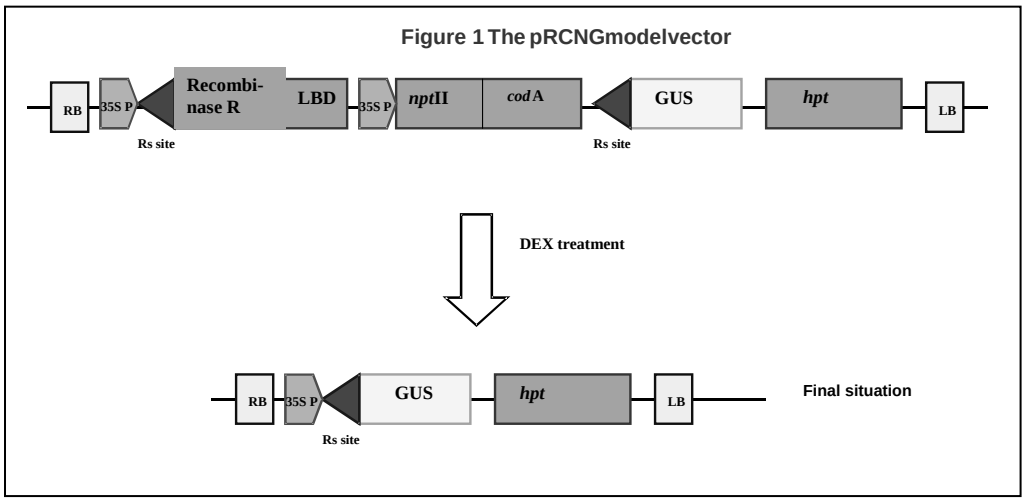


Fig. 1. A model vector, pRCNG, is presented showing all the aforementioned elements. In addition it contains the GUS coding region without a promoter. One Rs site is present between the R gene and the 35S promoter driving its expression. Upon recombination the 35S promoter is combined with the *gus* gene leading to GUS positive staining as a confirmation of the recombination event. A copy of the *hpt* gene outside the Rs sites in this model vector allows further use of positive selection when required.

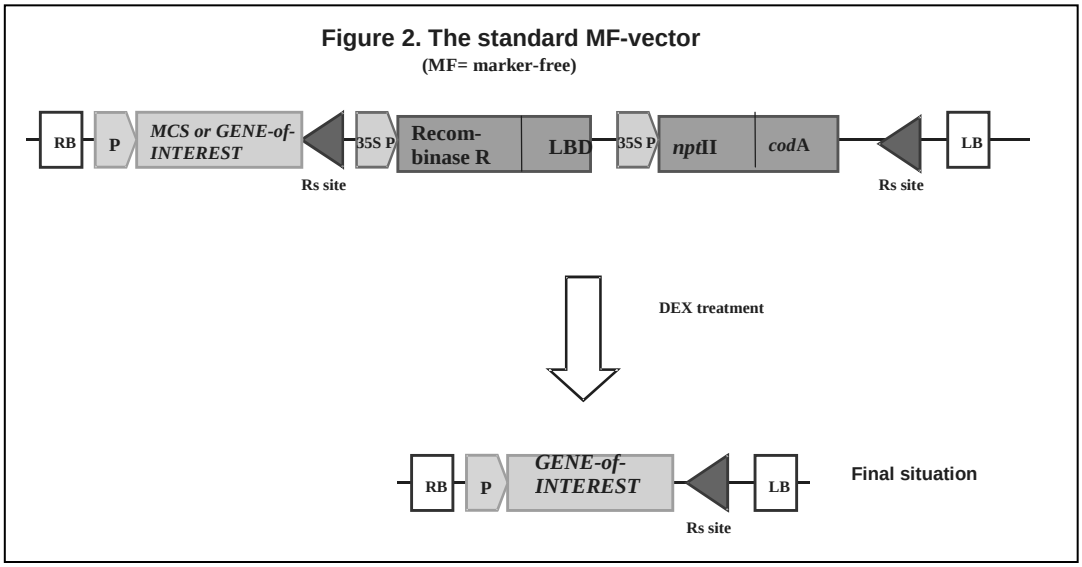


Fig. 2. A 'standard' vector has been assembled with only the elements needed, now including promoter-driven R expression between the Rs sites and a multiple cloning site (MCS) outside them allowing introduction of any gene (expression) cassette leading to the trait of choice.