Info-sheet: **pMF-vectors**

pMF-vectors for marker-free technology

pMF is a new optimized plant transformation vector produced by Plant Research International, Wageningen for generation of genetically modified plants which are free from undesired DNA-sequences.
Use of the pMF vectors for plant transformation enables effective removal of undesired DNA sequences, like antibiotic resistance genes, from the obtained transgenic plants or plant tissues. Removal of selectable marker genes may be desirable for the production of genetically modified crops with increased consumer acceptance. In addition, marker removal enables stacking of transgenes by recurrent transformations.

Principle

The pMF vector provides an inducible site-specific recombination system for removal of undesired DNA sequences. A negative selection step using the *cod* A (cytosine deaminase) gene, ensures the ultimate production of completely marker-free plants (Schaart *et al.* (2004). *Cod* A is a conditionally lethal dominant gene encoding an enzyme that converts non-toxic 5-fluorocytosine (5-FC) to cytotoxic 5-fluorouracil (5-FU).

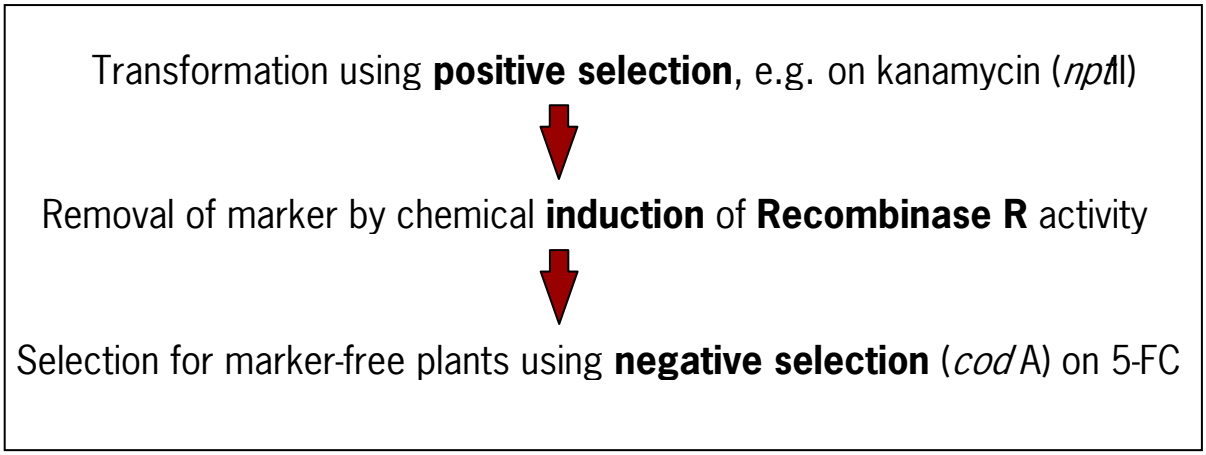


Figure 1. Selection scheme for producing marker-free transgenic plants using pMF1. A similar selection scheme can be followed using pMF2 or pMF3 vectors, when positive selection on either hygromycin or phosphinothricin, respectively, is preferred.

Chemically induced marker removal

The marker removal system (schemes in Figures 1 & 2) fits easily in existing *Agrobacterium* -mediated transformation protocols. After positive selection of transgenic tissue or plants, chemical induction of Recombinase R activity will result in elimination of DNA-sequences that are flanked by intact recombination sites (Rs). The Recombinase R protein is inactivated by the presence of the Ligand Binding Domain (LBD) of the glucocorticoid receptor. Activation of the Recombinase R protein activity is achieved by an overnight incubation of plant tissue in a 10 µM dexamethasone solution. Subsequent (negative) selection on 5-

FC should prevent development of plant tissue still containing the *cod* A-gene, thereby preventing the occurrence of chimeras due to incomplete DNA excision.

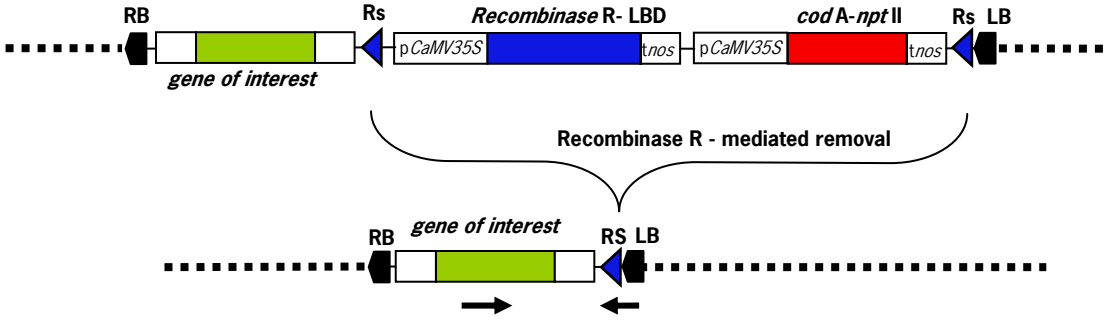
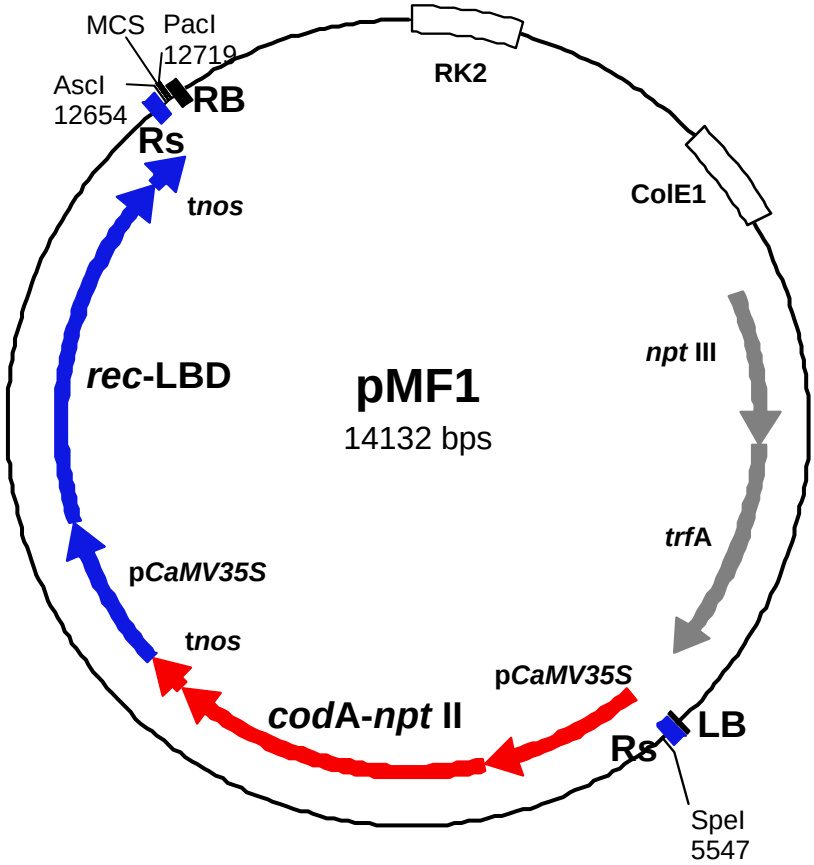


Figure 2. Schematic representation depicting the T-DNA of pMF1 before and after Recombinase R-mediated removal of the DNA-sequences that are flanked by the recombination sites (Rs). Black arrows indicate primer locations for PCR-check for confirmation of the recombination event.

Vector map

The pMF1 vector (Figure 3) is equipped with the *npt* II gene for kanamycin selection of transgenic shoots. Two additional pMF-versions, pMF2 and pMF3 are available in which the selection gene *hpt* (hygromycin resistance) and *pat* (phosphinothricin resistance), respectively, have been introduced into a unique *Spe* I-restriction site of pMF1. It is also possible to introduce reporter genes like *gus* or *gfp* at the *Spe* I-site for monitoring initial transformation efficiencies and as a visual marker for checking marker removal. All selection genes and reporter genes will ultimately be removed.



RB, LB, Right and Left Border of T-DNA, respectively
Rs, Recombination Site
***rec*-LBD**, Fusion of Recombinase R-gene and Ligand Binding Domain
***cod* A-*npt* II**, Fusion of *cod* A gene for negative selection on fluoro cytosine and *npt* II gene for positive selection on kanamycin
***Spe*I,** restriction site in which *hpt* II or *pat* genes have been inserted in pMF2 and pMF3, respectively
MCS, Multiple Cloning Site,

Figure 3. Vector map of pMF1

Ordering

The pMF-vectors are available by returning a signed version of the material transfer agreement (MTA). There a two different MTA's, one for 'not-for-profit' organizations and one for commercial organizations. Payment of a handling fee of € 995,-. allows the use of the pMF-vectors for research purposes only. Conditions for commercial exploitation of pMF-vectors are described in both MTA's.
It is also possible to order the pRCNG-model vector which is described in Schaart et al. (2004) by returning the corresponding MTA (please send a request to jan.schaart@wur.nl). With this vector, which is available for free, you can test the applicability of our marker-free technology to your favorite plant species.

Experimental data

So far, our marker-free technology has been tested in a limited number of plant species, including strawberry, apple and tobacco.

Transformations

We have observed no differences in transformation efficiencies as compared to transformations using pBinplus (van Engelen et al., 1995), a pBin19 derivative, which we normally use in our transformation studies. In quite a number of transgenic events we have noticed transfer of binary vector backbone sequences to the plant genome (ranging from 10 to 50%). The transfer of vector backbone sequences is a common feature in *Agrobacterium* mediated transformation systems. The frequency we have observed is similar to that reported in literature (see for example De Buck et al., 2000).

Induction of recombinase and negative selection

For elimination of marker-DNA we have studied timing of induction of recombination activity and application of negative selection (see Figure 1).
For early induction of recombinase activity we have treated plant tissue with dexamethasone one or two months after the start of the transformation experiment. At this time-point no regenerants were visible yet. Together with the induction of recombinase activity the positive selection on kanamycin was stopped and induction of regeneration was continued under negative selective conditions (addition of 5-FC).
In the alternative late marker elimination strategy, first transgenic plants were regenerated under continuously positive selection pressure. Then in a secondary step shoot tissue (or other appropriate explant types with regeneration capacity) from these plants was treated with dexamethasone for induction of recombinase activity and subsequently secondary regenerants were induced under negative selection conditions (using 5-FC in the regeneration media).
Both early and late induction strategies resulted in the recovery of completely marker-free transgenic plants. The early induction strategy resulted in marker-free plants quite effectively, but recovery of marker-free plants was less efficient than for the late induction strategy. Depending on the exact timing of early induction (and finishing positive selection) a considerable number of non-transgenic escapes were obtained.
The late induction strategy proved to be more efficient in recovery of completely marker-free transgenic plants. However, the longer period necessary for producing marker-free plants and the double regeneration step needed may render the late induction strategy less favorable.
Application of marker-free technology to *Nicotiana tabacum* resulted in an unexpectedly low number of marker-free plants. Transformation of pMF1 using the supervirulent *Agrobacterium* strain AGL0 resulted in high transformation efficiencies, but after induction of recombination (both early and late inductions) just a single marker-free transgenic plant was recovered. The possible integration of high T-DNA copy-number may have hampered marker-removal in this case. Alternatively, incomplete T-DNA transfer may have occurred, resulting in loss of one of the recombination-sites (Rs), which is located direct upstream of the left T-DNA border (LB). Loss of Rs prevents recombination. Recently, incomplete T-DNA transfer was found to be a common phenomenon in transgenic tomato (Thomas

and Jones (2007), and the authors suggested that the specific mechanism leading to incomplete T-DNA integration may be a common feature to Solanum species. Improvement of application of clean vector technology to *Nicotiana* will be subject of future studies.

Optimization of dexamethasone treatment and negative selection on 5-FC

For the induction of recombinase activity we usually perform an overnight incubation of the plant tissue in liquid MS-medium (including sugar; no hormones or selective agents, but *Agrobacterium*-eliminating agents such as cefotaxime may be included) containing 10 µM dexamethasone. After overnight incubation the plant tissue is transferred to fresh regeneration medium supplemented with 1 µM dexamethasone and 5-FC.

We have tested the effect of dexamethasone concentrations ranging from 1-50 µM on the regeneration capacity of non-transformed tissue without observing a negative effect. However, overnight incubation in liquid medium caused extreme expansion and vitrification (hyperhydricity) of the *Nicotiana* leaf explants during subsequent culture.

For negative selection against tissue that is still containing marker sequences (*cod* A) we usually apply 5-FC at a concentration of 150 mg/l. Testing higher 5-FC concentrations showed that for *Nicotiana* concentrations up to 1000 mg/l 5-FC had no negative effect on the regeneration capacity of non-transgenic leaf explants. Strawberry seemed to be more sensitive to 5-FC than tobacco. At 250 mg/l 5-FC a normal regeneration capacity was observed, but at 500 mg/l 5-FC and higher concentrations regeneration was prevented. Remarkably, for both strawberry and tobacco low 5-FC concentrations (100-150 mg/l) had a stimulatory effect on the regeneration capacity.

Testing for absence of marker sequences

We have designed a series of primer pairs to screen putative marker-free plants for 1.) absence of *Agrobacteria,* 2.) absence of vector backbone, 3.) occurrence of the recombination step and 4.) complete removal of all undesired sequences. A sheet with primer sequences will be send upon request.

For the PCR-check for the occurrence of the recombination step the reverse primer is located just behind the inserted gene of interest. An additional forward gene-of-interest-specific primer has to be designed for each new gene sequence to be transferred (see figure 2). The reverse primer is positioned on the overlap of the Rs-site and the LB, which prevents this primer from annealing to the other Rs-site which is located just behind the gene of interest in non-recombined sequences.

Patents

A patent application for our marker-free system is pending (US2004185567/EP1264891/WO02097102). Granted in NZ.

The Recombinase-LBD part falls under patent EP0632054, lapsed in SE, PT, DK, MC, LU, GR and NL.

References:

De Buck S, De Wilde C, Van Montagu M and Depicker A (2000) Determination of the T-DNA transfer and the T-DNA integration frequencies upon cocultivation of Arabidopsis thaliana root explants. *Mol Plant-Microbe Interact* 13: 658-665

Schaart JG, Krens FA, Pelgrom KTB, Mendes O and Rouwendal GJA (2004) Effective production of marker-free transgenic plants using inducible site-specific recombination and a bifunctional selectable marker gene. *Plant Biotechnology J.* 2: 223-240:

Thomas CM and Jones JDG (2007) Molecular analysis of *Agrobacterium* T-DNA integration in tomato reveals a role for left border sequence homology in most integration events. *Mol Gen Genomics* 278: 411-420.

van Engelen FA, Molthoff JW, Conner AJ, Nap JP, Pereira A and Stiekema WJ (1995) pBINPLUS – an improved plant transformation vector based on pBIN19. *Transgenic Res.* 4: 288–290.