Transgenic Plant Journal ©2007 Global Science Books

GLOBAL SCIENCE BOOKS

Osteospermum ecklonis Marker-Free Transgenic Plants: Analysis of Insertion by AL-PCR

Marina Laura • Giafar Safaverdi • Andrea Allavena*

C.R.A.- Unità di ricerca per la floricoltura e le specie ornamentali, Corso Inglesi 508, 18038 Sanremo (IM), Italy

***Corresponding author*: * a.allavena@istflori.it**

ABSTRACT

The generation of "marker-free" plants is a response to public concern and technical reasons (preclusion to use the same gene during further steps of transformation and gene silencing consequent to marker gene pyramiding by conventional breeding). A further step towards breeding of an advanced generation of transgenic plants is to avoid the insertion of DNA not essential for the correct expression or silencing of the useful transgene(s). To accomplish these purposes, transformation vectors derived from pGreen II 0000 were prepared. The original T-DNA of 814 bp was completely replaced with a new T-DNA sequence of 44 bp containing exclusively multiple cloning sites; *rolC* and *rolABC* were then cloned into the *Hind* III/*Eco* RI and *Eco* RI sites, respectively. Following *Agrobacterium*-mediated transformation of the ornamental species *Osteospermum ecklonis* (DC.) Norl. (Cape daisy) and screening of regenerated shoots by PCR amplifying the *rolA* and *rolC* gene fragments, three plant clones that had at least one complete copy of the *rolABC* genes, and which expressed the transcript and showed the expected phenotype (high number of branches, profuse flowering, compact plant habit) were identified. Adaptor ligation PCR was performed on these plants and several products were identified at both sides of the insert. Sequencing of amplification products revealed a complicated scenario far from the classical type of integration reported for *Agrobacterium*. Integration cases identified were classified as: correct integration, miss-recognition of left border (LB) and right border (RB) or rearrangements of the T-strand. None of the three marker-free plant clones expressing *rol ABC* had integrated a single copy of the genes. In addition, backbone sequences were found in all plants. These data indicate that these materials need further steps of conventional breeding to select progenies that had integrated the transgene(s) by inserting the minimum amount of DNA required for its correct expression. Strategies to improve the correct recognition of LB as T-strand terminations are finally discussed based on current knowledge.

Keywords: backbone integration, cape daisy, genetic engineering, ornamental plants, *rol* genes, T-DNA junction, transformation vectors

INTRODUCTION

The risk and damage that selectable marker genes may cause, either to human health or to the environment, even if not documented by scientific evidence, is constantly raised by public opinion. In Europe, following the Directive 01-18-EC, the presence of the most popular class of marker genes conferring resistance to antibiotics in field-grown transgenic plants, will be no longer allowed from 31st December, 2008. Moreover, the presence of a selectable marker gene in a transgenic genotype will preclude the use of the same gene during further steps of transformation; pyramiding multiple copies of marker genes by crossing may activate silencing. A response to this public concern and to address technical demands is the generation of "marker-free" plants. A further step towards breeding of an advanced generation of transgenic plants is to avoid the insertion of DNA sequences (ancillary sequences, plasmid sequences, aborted copies of the transgene, recombination sequences, etc.) not essential for the correct expression or silencing of the useful transgene.

Several methods are currently available for the production of marker-free transgenic plants: A) co-transformation and segregation (McKnight *et al*. 1987; de Block and Debrouwer 1991; Komari *et al*. 1996; Daley *et al*. 1998; Dalton *et al*. 1999); B) site-specific (Dale and Ow 1991; Gleave *et al*. 1999; Zuo *et al*. 2001) and intrachromosomal recombination (Zubko *et al*. 2000); C) transposable elements repositioning and elimination (Goldbrough *et al*. 1993; Ebinuma *et al*. 1997); D) transformation with the useful gene only. The last method does not require the preparation of complex constructs. If *Agrobacterium tumefaciens* transformation is chosen, a hypervirulent strain such as AGL0 or AGL1 (Lazo *et al*. 1991), carrying a plasmid with a minimum T-DNA and well recognized borders is strongly recommended.

The advantage of *Agrobacterium*-mediated transformation is believed to be the accuracy and efficiency of T-strand excision and integration into the plant genome. The whole T-DNA is generally integrated together with border sequences (Mayerhofer *et al*. 1991; van der Graaff *et al*. 1996). Sometimes the integrated T-DNA copy may either be truncated before the border; or may comprise vector DNA (Herman *et al*. 1990; Mayerhofer *et al*. 1991; Cluster *et al*. 1996) because of skipping the left border sequence during T-strand formation (Martineau *et al*. 1994). In addition, short sequence (fillers) of unknown origin may be found at the junction site (Gheysen *et al*. 1991; Gorbunova and Levy 1999).

We reported here our results to assemble *Agrobacterium* vectors suitable for the production of either "marker-free" or "clean" transgenic plants. In addition, we report the production of "marker-free" transgenic plants in the ornamental species *Osteospermum ecklonis* (DC.) Norl. (Cape daisy) and preliminary analysis of integrated DNA flanking regions by adaptor ligation PCR (AL-PCR).

The *rolC* and *rolABC* genes (Spena *et al*. 1987) that modify several ornamental traits in *O. ecklonis* (Giovannini *et al*. 1999) were used for this work.

Received: 9 August, 2007. Accepted: 30 October, 2007.

Original Research Paper

MATERIALS AND METHODS

Reagents and kits

All restriction and DNA modifying enzymes were from New England Biolabs™, (USA). The oligonucleotides and the primers were purchased from TIB MOLBIOL S.r.l. (Italy). Taq polymerase and Elongase Enzyme Mix were from Invitrogen (Paisley, United Kingdom). The following kits provided by QIAGEN (Germany) were used: "QIAprep Spin Miniprep Kit" for plasmid extraction; "QIAquick Gel Extraction Kit" for DNA elution from agarose gel; "QIAquick spin column" for enzyme inactivation and DNA purification; "Dneasy Plant Mini Kit" for plant DNA extraction; "Rneasy Plant Mini Kit" for RNA extraction. Contaminating DNA was eliminated in RNA samples using Sigma's Amplification Grade Deoxyribonuclease I. The two-step RT-PCR reactions, of Sigma Enhanced Avian RT-PCR Kit (Sigma-Aldrich S.r.l., Italy), were used for reverse-transcriptase reactions and for cDNA amplification.

Bacterial strains

All the molecular cloning experiments were carried using *Escherichia coli* strain (Max Efficiency® DH5α™ Competent Cells, Invitrogen, USA). The transformation was performed routinely by following the procedure of Hanahan (1983).

Plasmids were transferred into *A. tumefaciens* strain AGL1 (Lazo *et al*. 1991) containing the helper plasmid pSoup, which is essential for replication of pGreen (Hellens *et al*. 2000), using a freeze-thaw method (An 1987).

Isolation of *rolC* and *rolABC* genes

The *rolC* and *rolABC* genes were isolated from plasmid pPVC002 *rolC* and pPVC002 *rolABC* (Spena *et al*. 1987), by digestion, using *Hind* III-*Eco* RI and *Eco* RI, respectively.

Construction of pGreen *rolC*

The recipient plasmid used in this experiment was pGreen 0000 that contains in the T-DNA unique restriction sites within a β-galactosidase gene (Hellens *et al*. 2000). The *rolC* gene was cloned into the *Hind* III-*Eco* RI sites of the plasmid polylinker. Plasmids pGreen *rolC* was then moved into *A. tumefaciens* AGL1 containing the helper plasmid pSoup (Laura *et al*. 2003).

Construction of pGreen II *rolC* and pGreen II *rolABC*

Plasmid pGreen II 0000, used in this experiment, is a modified version of pGreen 0000 with improved DNA stability (www.pgreen.ac.uk).

The Construction of pGreen II adaptor

The T-DNA of pGreen II 0000 was excised by digestion with *Stu* I and *Acl* I and replaced with a shorter adaptor polylinker sequence. The adaptor sequence was generated annealing two complementary oligonucleotides: (5′-CGTTAACAAAGCTTGGGACCGCGGTGGAATTCCAGCTATGAAGG and 5′-CCTTCATAGCTGGAATTCCACCGCGGTCCCAAGCTTTGTTAA). The adaptor contains: Two internal restriction sites (*Hind* III and *Eco* RI) underlined in the above sequences; 5′ terminal blunt sequence complementary to plasmid blunt sequence generated by *Stu* I and 3′ terminal protruding sequence complementary to the plasmid protruding sequence generated by *Acl* I. pGreen II adaptor was generated by ligation of the adaptor with the pGreen II plasmid previously digested.

Insertion of *rolC* and *rolABC* in pGreen II adaptor

The *rolC* and *rolABC* genes were cloned into the *Hind* III-*Eco* RI and *Eco* RI sites of pGreen II adaptor, respectively. The arrangement of *rolABC* genes insertion from LB to RB was checked by construct digestion with *Hpa* I. Plasmids pGreen II *rolC* and pGreen II *rolABC* were moved into *A. tumefaciens* AGL1 containing the helper plasmid pSoup.

Plant material and transformation

Leaves of *in vitro*-grown *O. ecklonis* plants (clone DM005) were used in transformation experiments, following the method proposed by Giovannini *et al*. (1999).

Three transformation experiments were performed, using *A. tumefaciens* AGL1 containing pGreen *rolC* plasmids (experiment A) and pGreen II *rolABC* plasmids (experiments B1 and B2).

Leaf pieces were wounded and immersed in the bacterial culture. One hundred and fifty explants were inoculated with bacteria in each experiment. After 30 minutes, the explants were blotted on sterile filter paper briefly and placed on co-cultivation medium for 2 days. Co-cultivation medium was composed of MS macro- and microelements (Murashige and Skoog 1962), MS vitamins, sucrose 30 g/L, 3-indolyl-acetic acid (IAA) 2 mg/L, 6-benzylaminopurine (BAP) 1 mg/L, agar 8 g/L. After 2 days, the leaf pieces were blotted and placed on regeneration medium (co-cultivation medium with 100 μg/ml Cefotaxime) to contain *Agrobacterium* growth. Non-inoculated explants were prepared as a control and grown directly on co-cultivation medium.

Regenerated shoots, 1 to 1.5 cm long, with a clear independent origin on the leaf explants, were excised and cultivated on propagation medium (MS salts and vitamins, sucrose 30 g/L and agar 8 g/L) plus Cefotaxime (100 μg/ml). Each shoot representing a separate regeneration event was subsequently propagated through axillary shoot proliferation to provide plant clones. All plant cultures were maintained in a growth room at 23-25°C with 16 h light photoperiod. *In vitro* plants were acclimatized under mist and grown in a containment greenhouse.

Analysis of regenerated plants

Amplification of the transgenes

The presence of foreign genes in the regenerated plants was evaluated by PCR (Hamill *et al*. 1991). Genomic DNA was extracted from 100 mg of leaves of transgenic and control *O. ecklonis in vitro* grown plants. All the plants of experiment A were screened for the presence of the *rolC* gene by PCR. Plants of experiments B1 and B2 were checked first for *rolA*, that is close to LB and consequently is less likely inserted into the plant genome, and then for *rolC* (**Fig. 1**).

Amplification of *rolA* gene (experiments B1 and B2) was checked using the following primers: (5′-GACGTCATTGCACTCGTCAC) as forward and (5′-TTGATTGCGGATTTCCTTTC) as reverse to amplify a 199 bp fragment. The following cycling condition were used: 2 min at 94°C, 30 cycles 1 min at 94°C, 1 min at 60°C, 3 min 72°C and a final extension of 10 min at 72°C.

Forward primer (5′-CGACCTGTGTTCTCTCTTTTTCAAGC) and reverse primer (5′-GCACTCGCCATGCCTCACCAACTCACC) were designed and used for the amplification of a 514 bp fragment of the *rolC* gene. PCR reactions were performed in a thermocycler PTC 100 (MJR Research Inc., USA), using the following cycling program: 2 min at 94°C, 30 cycles 1 min at 94°C, 2 min at 60°C, 3 min 72°C and a final extension of 10 min at 72°C.

The absence of *A. tumefaciens* contamination in the plants that were positive in PCR analysis of the *rol* genes, was checked by PCR amplification of a 326 bp fragment of the *virC1* gene. The sequence of *virC1*-specific primers (PVIRC2775 and PVIRC3101) and PCR conditions were reported by Vaira (1995).

A fragment of 4000 bp, spanning the entire *rolABC* ORF, was amplified with a forward primer (5′-CACATCTCCCGAAAAATGCT) and a reverse primer (5′-AAAGCGCGATGAAATCAAGT) in the clones 426, 428 and 441 to verify, in each of them, the presence of at least one full-length copy of the insert containing all three genes. The following parameters were used: 3 min denaturation at 94°C, followed by a 40 cycles of 30 s at 92°C, 30 s annealing at 55°C, 2 min elongation at 72°C and a final extension at 72°C for 3 min.

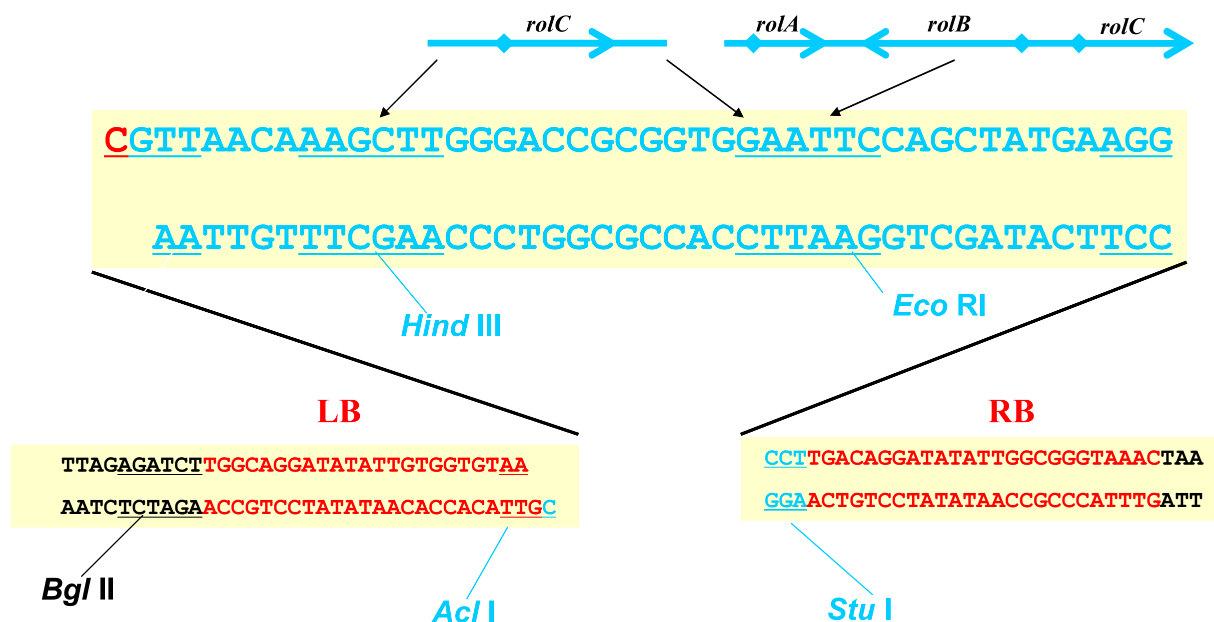

Fig. 1 The pGreen adaptor vector: a polylinker of 44 bp, cloned in *Acl* I and *Stu* I sites, replaces the original T-DNA of pGreen II 0000. The *rolC* gene and *rolABC* gene were cloned into the *Hind* III-*Eco* RI and *Eco* RI sites, respectively.

Expression analysis

The phenotype of *rolABC* putative transgenic plants, acclimatized in greenhouse, was characterized for their plant habit according to Giovannini *et al*. (1999).

Expression of the *rolC* gene was investigated by RT-PCR on plants positive to amplification of all three *rol* genes. Two plants of experiment A (one of clone 2 with the characteristic phenotype and one of clone 31 with a normal phenotype) and 3 plants of experiment B1 (all with characteristic phenotype) were analysed together with parent plants.

Identification of junctions

Adaptor ligation PCR (AL-PCR) was performed on three *rolABC* marker-free transgenic plants of clone 426, 428, 441 and a control plant, following the procedure proposed by Zheng *et al*. (2001). Total DNA was isolated from 100 mg of leaves. Genomic DNA samples of 2.5 µg were restricted separately by *Pvu* II, *Rsa* I and *Sca* I in a final volume of 100 µl to generate blunt-end fragments, then the enzymes were heat inactivated at 65°C for 10 min and removed by purification. An asymmetric adaptor was prepared by annealing two complementary oligonucleotides (ADAPL and ADSPS) and then ligated to genomic restricted DNA, as described by Cottage *et al*. (2001). A single-copy reconstructed positive control, hypothetically corresponding to a single T-DNA integration in the *O. ecklonis* genome, was prepared by mixing 1 µg of genomic DNA from an untrasformed control plant with 10 pg of pGreen II *rolABC* vector DNA (4.5 kbp), digested with *Rsa*I and processed as before.

Two successive PCRs were carried out to avoid non-specific PCR amplifications using two pairs of primers designed on adaptors (AP1 and AP2) and on T-DNA (WP1 and WP2), for each T-DNA border. The adaptor primer AP1 and the nested adaptor primer AP2 were designed as by Zheng *et al*. (2001). The walking primer for amplification of the left border junction (LB1a: 5′-GAGTTGCGTGGCCAGTTAATAC) and the nested walking primer (LB2c: 5′-AGCACGTGAGAGACAAGTAAAT), were complementary to the T-DNA sequence located from 217 to 190 bp and from 156 to 129 bp, respectively. Similarly, the walking primer for amplification of the right border junction (RB1: 5′-ATGG AGCTAACTTCGACAAAGG) and the nested walking primer (RB2c: 5′-GGGCCACAATATTTGACCTATATGC), were complementary to the T-DNA sequence located from 3880 to 3904 bp and from 4252 to 4278 bp, respectively.

Amplification was performed on the base of the conditions described by Zheng *et al*. (2001).

With the aim of identifying longer junctions, the following amplification conditions were tested. The primary PCR was carried out with 1 µl library, 1 µl Elongase Buffer A, 4 µl Elongase Buffer B, 0.5 µl Elongase Enzyme Mix, 1 µl dNTPs (10 mM), 1 µl AP1 primer (10 µM), 1 µl WP1 primer (10 µM), 15.5 µl of water were added to give a final volume of 25 µl. The following cycling conditions were used: 30s at 94°C, 35 cycles 30s at 94°C, 30s at 59°C, 7 min 68°C and a final extension of 15 min at 68°C. Secondary PCR amplification was performed using 1 µl of the primary PCR amplificate diluted 1 to 100, 1 µl AP2 primer, 1 µl WP2 primer and the other components used for primary PCR reaction. The cycle conditions were as before, except that annealing was performed at 65°C.

Twenty microlitres of the PCR products were separated on a 1% agarose gel. Some of the observed bands were eluted and sequenced by BMR-Genomics (CRIBI, Padova, Italy).

RESULTS AND DISCUSSION

Construction of new plasmids

The plasmid pGreen *rolC* does not contain antibiotic resistance genes on the T-DNA and is thus suitable for "marker-free" transgenic plant production via co-transformation or transformation with the useful gene only (McKnight *et al.* 1987; de Block and Debrouwer 1991; Komari *et al.* 1996; Daley *et al.* 1998; Dalton *et al.* 1999; Vetten *et al.* 2003). The original T-DNA of pGreen 0000 and of pGreen II 0000 contains the multiple cloning sites within the *LacZ* gene and additional plasmid sequences. This T-DNA of 814 bp was completely replaced with a new T-DNA sequence of 44 bp in the pGreen II adaptor (**Fig. 1**). The new T-DNA contains exclusively multiple cloning sites, therefore the pGreen II adaptor can be considered a "clean" vector, suitable for further cloning. The pGreen II *rolC* and the pGreen II *rolABC* are the first derivative.

Plant transformation

Plenty of shoots were regenerated in the absence of selective pressure in the medium, following co-cultivation of leaf explants of *O. ecklonis* with *A. tumefaciens* AGL1 carrying either pGreen *rolC* or pGreen II *rolABC* (**Fig. 2**). Overall, more than 150 shoots, with clearly independent origin, were isolated from the leaf explants co-cultivated in the two experiments and propagated separately to establish plant clones.

Fig. 2 ***In vitro*** **grown** ***O. ecklonis*** **leaf explants regenerating plenty of shoots, in the absence of selective pressure in the medium, following co-cultivation with** ***A. tumefaciens*** **AGL1 carrying pGreen II** ***rolABC*****.**

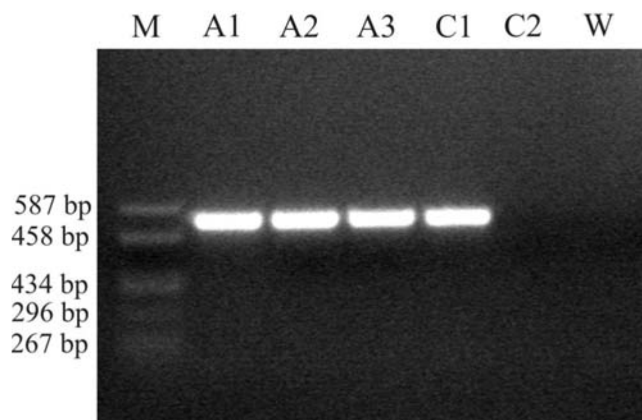

Fig. 3 The expected 514 bp ***rolC*** **fragment amplified by PCR in three plants, selected from independent event of regeneration, following co-coltivation of** ***O. ecklonis*** **leaf explants with pGreen II** ***rolC*** **(A1, A2 and A3) and in the positive control (C1: plasmid pGreen II** ***rolC*****).** M: molecular marker (pUC18 DNA *Hae* III digest), C2: untransformed plant, W: no template.

Analysis of regenerated plants

Among the 50 clones of plants obtained following co-cultivation with *A. tumefaciens* carrying pGreen *rolC* (experiment A), 33 randomly selected clones were analysed by PCR (**Table 1**).The expected 514 bp *rolC* band (**Fig. 3**) was amplified from seven plant clones (21%). PCR amplification of a 326 bp fragment of the *virC1* gene showed that only one plant out of seven was contaminated by the *Agrobacterium*. Six plants (18%) were then expected to be of transgenic nature (**Fig. 4**).

Two of the six putative transgenic clones (6% of the analysed plant clones), acclimatized in greenhouse, showed the characteristic phenotype of plants expressing *rolC* such as short internodes, erect branches, profuse branching, slightly pale green leaves, compact plant habit as reported by Giovannini *et al*. (1999). The other clones evidenced a phenotype similar to control plants or intermediate. The expected RT-PCR product, confirmed the *rolC* expression in the plants with the characteristic *rolC* phenotype only.

In experiments B1 and B2, performed with the vector pGreen II *rolABC*, 40 and 28 randomly selected plants, respectively were analysed by PCR (**Table 1**). The expected 199 bp *rolA* fragment was amplified from 11 plant clones in each experiment. Ten and nine plants out of 11 (*rolA*-positive) were also positive in *rolC*-PCR analysis in the experiments B1 and B2, respectively. PCR amplification of a 326 bp fragment of the *virC1* gene showed that only one plant out of 10 was contaminated by *Agrobacterium* (experiment B1). Therefore, the frequency of putative transgenic plants was 22.5% and 32% in the two experiments. Five of the 9 transgenic clones of experiment B1 (12.5% of the analysed plant clones), acclimatized in greenhouse, showed the characteristic phenotype of plants expressing *rolABC* (**Fig. 5**): high number of branches per plant, short internodes, compact plant habit and profuse flowering which is in agreement with previous report (Giovannini *et al*. 1999). Expression of *rolC* gene (RT-PCR) was confirmed in three plants (clones 426, 428, 441) amplifying *rolA* and *rolC* by PCR and with a typical *rolABC* phenotype as reported before. In the same plants, the expected fragment of 4000 bp, spanning the *rolA*, *rolB*, and *rolC* ORFs, was amplified demonstrating that at least one full-length copy of the genes was integrated. An additional product of about 8000 bp was found in clone 428, signifying likely the occurrence of a tandem copy of the *rolABC* genes or T-DNA rearrangements.

Since *rolABC* are hormone genes that change plant development (Schmulling *et al*. 1988), their expression in the plants can play a role in cell differentiation and confer a

Fig. 4 Phenotype of an ***in vitro*** **grown transgenic plant that express** ***rol C*** **gene (right) in comparison with a normal regenerated plant (left).** The profuse branching and rooting of the transgenic plant represent an extreme phenotype not widespread in all transgenic plant clones.

positive selection to transgenic cells in some species and genotypes during cell division and differentiation. In addition, phenotypes conferred by *rolC* and *rolABC* allow early selection of putative plant clones *in vitro* and *in vivo*. *RolC* alone or in combination with *rolA* and *rolB* may be proposed either as selectable or visible markers especially in floricultural species where the pleiotropic effect of *rol* genes may be valuable. Vetten *et al*. (2003) reported that co-cultivation of potato explants with the *A. tumefaciens* strain AGL0 allowed the recovery of transgenic potato shoots at a frequency of 1 to 5% in the absence of selection. Moreover, transgenic somatic embryos of cassava were recovered at a frequency of 2% following co-cultivation with *A. tumefaciens* strain AGL1. AGL1 is a $RecA^-$ version of AGL0 (Lazo *et al*. 1991). The satisfactory frequency of transformation in both Vetten's and our experiments may then be ascribed to the hyper-virulence of the plasmid pTiBo452

Table 1 Analysis of regenerated plants in independent transformation experiments using pGreen constructs carrying *rolC* (A) and *rolABC* genes (B1 and B2).

Exp.	Analysed plants (n°)	PCR *rolA*[1]	PCR *rolC*	PCR *virC*[2]	Putative transgenic plants[4]	Phenotypic expression[5]	Phenotypic expression[6]
A[7]	33	-	7/33[1]	1/7	6/33 (18%)	2/6 (33%)	2/33 (6%)
B1 This work	40	11/40 (27%)	10/11[3]	1/10	9/40 (22.5%)	5/9 (55%)	5/40 (12.5%)
B2 This work	28	11/28 (39.28%)	9/11[3]	0/9	9/28 (32%)	n.d.	n.d.

[1]positive/ tested plants; [2]positive/ *rolC* positive; [3] positive/ *rolA* positive; [4]*rolC* positive/ regenerated plants; [5]*rol* phenotype/ *rolC* positive; [6]*rol* phenotype/ regenerated plants; [7]Laura *et al*. (2003); n.d.: not detected.

Fig. 5 Plant phenotype of three clones expressing *rolABC* (left) in comparison with a normal plant (right). All plants were grown in greenhouse. The clones represent various level of expression of the characters influenced by *rolABC*: number of branches per plant, internodes length, plant compactness and profusion of flowering.

Table 2 Fragments amplified by AL-PCR following PCR conditions 1 described by Zheng *et al*. (2001) and PCR conditions 2 (Elongase Enzyme Mix amplification protocol) to identifying longer junctions.

Transgenic Clones	PCR conditions 1						PCR conditions 2					
	LIBRARY						LIBRARY					
	***Rsa* I**		***Sca* I**		***Pvu* II**		***Rsa* I**		***Sca* I**		***Pvu* II**	
	RB	**LB**	**RB**	**LB**	**RB**	**LB**	**RB**	**LB**	**RB**	**LB**	**RB**	**LB**
426	300 bp	300 bp*	500 bp 300 bp	-	300 bp* 150 bp*	-	1600 bp 700 bp 500 bp 400 bp 300 bp*	500 bp* 400 bp	1000 bp 700 bp 300 bp*	300 bp	2000 bp* 1600 bp* 1000 bp 700 bp	900 bp 600 bp*
428	500 bp* 200 bp	400 bp	1000 bp* 600 bp 400 bp 200 bp	400 bp	1000 bp 500 bp* 200 bp	700 bp 500 bp	-	-	-	-	2000 bp 1600 bp	900 bp 600 bp
441	1000 bp 800 bp 500 bp 300 bp	-	300 bp 200 bp	300 bp*	1000 bp 500 bp* 250 bp	-	1200 bp 1000 bp 900 bp 790 bp* 530 bp*	800 bp* 700 bp 600 bp	-	-	-	1200 bp

* Sequenced fragment

present in both strains. Recently a significant improvement of tobacco transgenic plant recovery was reported by Jia *et al*. (2007) by a procedure involving a vacuum treatment to leaf explants immersed in the bacterial suspension. Fifteen percent of the regenerated shoots were PCR-positive and 7.2% showed the phenotype conferred by the GUS gene, which was used as marker. These frequencies are comparable to those found in our work. For species with good regeneration ability and highly susceptible to *Agrobacterium* infection, the opportunity to breed marker-free transgenic plants may became routine playing either on strain virulence and infection procedure.

Identification of junctions

Three *rolABC* marker-free transgenic plants, an untransformed plant (as a negative control) and one copy reconstructed positive control were analysed by AL-PCR for the detection of junctions at the RB and LB regions. The number and size of amplification products in transgenic plants are summarized in **Table 2**. Following the procedures of Zheng *et al*. (2001), amplification products ranging from 150 bp to 1000 bp were identified. As expected, the Elongase Enzyme Mix amplification protocol allowed the identification of longer fragments ranging from 300 bp to 2000 bp. Employing the two amplification protocols five products only at RB were of a comparable size, likely representing the same product. Seventeen products were amplified at the LB in comparison with a significantly higher number (35) at the RB. Amplification products were not detected in the negative control. Products of the expected size (1600 bp at RB and 550 bp at LB) were obtained with the positive control. Both libraries and amplification protocols seem fundamental to provide an overview of the insertion events.

Some of the amplification products were sequenced. Data are reported in **Table 3**. Analysis of insertion events revealed a complicated scenario, very different from the canonical type of insertion in the plant genome driven by *Agrobacterium*: in addition to the T-DNA, 3 nucleotide only of the RB (TGA in nopaline strains derived vectors) and the LB missing the TGG residue are inserted (Kim *et al*. 2003). Integration cases identified in this work might be classified as: correct integration; miss-recognition of LB and RB; rearrangements of the T-strand. Correct integrations are a minority of the sequenced plant junctions. At RB, junctions 11 and 12 show the typical 3 base residue due to an expected nick, between the third and fourth base during T-strand initiation. In five cases (4, 6, 7, 8 and 10) 8 to 50 T-DNA bases are missing before the RB. Such amount of missed bases does not interfere with the ORF of the *rolABC* genes. Missing of the typical 3 base RB residue and a similar amount of T-DNA bases was observed in both monocotyledonous barley and rice (Stahl *et al*. 2002; Kim *et al*. 2003) and dicotyledonous aspen (Kumar and Fladung 2002) and *Arabidopsis thaliana* (Brunaud *et al*. 2002). The LB junctions 14 and 15 show a two- base deletion in addition to the expected skips of the TGG residue.

Table 3 Sequence of the amplification products of *Osteospermum* clones 426, 428 and 441 identified at RB and LB by AL-PCR from libraries *Pvu* II, *Rsa* I and *Sca* I.

Library	Fragment number (bp)	Sequences
RB 426 *Pvu* II	1 (2000)	GGGCCACAATATTTGACCTATATGCACTGGCCCACGCGCCGCAATAGATGAAAATTGCCAAAATTAGCTATCGGTCTTCTGAAAAGAAGGGCCGACATGTTTTCATAGACCATGCAAAGTCATACTACCTGAAACTGATAAATAACGACAAAGAAAGTAGCCTATTTAAAAGTCGCTATAGCATGAATTCCAGCTATGAAGGCCTGGCGGGACATGTTGCGAGTGAAACGTTTTCTCTGTATGTGTTTGTTTGAGATCTCTTGAGAGCTAGAGGCCTGCCAAGNGCCTGGATTATGACAAATGTCCTGTTGAACTATAATATCATTAGGCCCCGCCCCNCTGNNTTGTTGATACTGCACTATATGTGTAGCAGATTAGATAAACCTA...
	2 (1600)	GGGCCACAATATTTGACCTATATGCACTGGCCCACGCGCCGCAATAGATGAAAATTGCCAAAATTAGCTATCGGTCTTCTGAAAAGAAGGGCCGACATGTTTTCATAGACCATGCAAAGTCATACTACCTGAAACTGATAAATAACGACAAAGAAAGTAGCCTATTTAAAAGTCGCTATAGCATGAATTCCAGCTATGAAGGCCTTGACAGGATATATTGGCGGGTAAACTAAGTCGCTGTATGTGTTTGTTTGAGATCTCATGTG...CAATATTTTCACACCTGCCCGGGCCGTCGACCACGCGTGCCCTATAGT
	3 (300)	GGGCCACAATATTTGACCTATATGCACTGGCCCACGCGCCGCAATAGATGAAAATTGCCAAAATTAGCTATCGGTCTTCTGAAAAGAAGGGCCGACATGTTTTCATAGACCATGCAAAGTCATACTACCTGAAACTGATAAATAACGACAAAGAAAGTAGCCTATTTAAAAGTCGCTATAGCATGAATTCCAGCTATGAAGGCCTTGACAGGATATATTGGCGGGTAAACTAAGTCGCTGTATGTGTTTGTTTGAGATCTCATGTGAGCAAAAGGCCAGCAAAAGCCAGACCTGCCCGGGCCGTCGACCACGCGTGCCCTATAGT
	4 (150)	GGGCCACAATATTTGACCTATATGCACTGGCCCACGCGCCGCAATAGATGAAAATTGCCAAAATTAGCTATCGGTCTTCTGAAAAGAAGGGCCGACATGTTTTCATAGACCATGCAAAGTCATACTACCTGAAACTGATAAATAACGACAAAGAAATTACCACGCGTGCCCTATAGT
RB 426 *Rsa* I	5 (300)	GGGCCACAATATTTGACCTATATGCACTGGCCCACGCGCCGCAATAGATGAAAATTGCCAAAATTAGCTATCGGTCTTCTGAAAAGAAGGGCCGACATGTTTTCATAGACCATGCAAAGTCATACTACCTGAAACTGATAAATAACGACAAAGAAATTTCCACGCCGGAGATGATATCGAATATGTTCTGTTAAGTTAAAATAAGCTGCGAGCCATGGCGCGATTGTCCTGTTTTATTAATATAGTACCTGCCCGGGCCGCGACCACGCGTGCCCTATAGT
RB 426 *Sca* I	6 (300)	AGATGAAAATTGCCAAAATTAGCTATCGGTCTTCTGAAAAGAAGGGCCGACATGTTTTCATAGACCATGCAAAGTCATACTACCTGAAACTGATAAATAACGACAAAGAAAGTAGCCTATTTAAAAGTCGCTATAGCATGAATTCCAGCTATGTAGAACTTGATGACCCAGCCATAATTAAGGNGACAGAACAA
RB 428 *Pvu* II	7 (500)	AAATTGCCAAAATTAGCTATCGGTCTTCTGAAAAGAAGGGCCGACATGTTTTCATAGACCATGCAAAGTCATACTACCTGAAACTGATAAATAACGACAAAGAAAGTAGCCTATTTACCTGCCCGGGCCGTCGACCACGCGTGCCCTATAGTAATCACTAGTGCGGCCGCCTGCAGGTCGACCATATGGGA...
RB 428 *Rsa* I	8 (500)	GGGCCACAATATTTGACCTATATGCACTAGCCCACGCGCCGCAATAGATGAAAATTGCCAAAATTAGCTATCGGTCTTCTGAAAAGAAGGGCCGACATGTTTTCATAGACCATGCAAAGTCATACTACCTGAAACTGATAAATAACGACAAAGAAAGTAGCCTATTTACCTGCCCGGGCCGTCGACCACGCG...
RB 428 *Sca* I	9 (1000)	AAATTGCCAAAATTAGCTATCGGTCTTCTGAAAAGAAGGGCCGACATGTTTTCATAGACCATGCAAAGTCATACTACCTGAAACTGATAAATAACGACAAAGAAAGTAGCCTATTTAAAAGTCGCTATAGCATGAATTCCAGCTATGAAGGCCTTGACAGGATATATTGGCGGGTAAACTAAGTCGCTGTATGTGTTTGTTTGAGATCTCATGTGAGCAAAAGGCCAGCAAAAGGCCAGACCTGCCCGGGCCGTCG...
RB 441 *Pvu* II	10 (500)	...TGATAAATAACGACAAAGAAAGTAGCCTATTTAAAAGTCGCTATAACCTGCCCGGGCCGCCGACCACGCGTGCCCTATAGTAATCACTAATGCGGCCGCCTGCAGGTCAACCATATGGGAGAGCTCCCAACACCTTGGATGCATAACTTGAGTATTCAATAATGTCACCTAAATAGCTTGGAGTGATACTGGTCTTGGCCGTGTTTCCCATGGGAACTTGTTATCGCAATAGAATTCCACACCAAAACGAGCCGGAATCATAAGAAAAAAAGCGTGGGCACGTATCATGGACCATGCAAAGTCAACTACGTTAAACTCATAAACAACGTCACAGTAAGTAGCCCAGTCAACCAGCTCATAGACCGTCCGG
RB 441 *Rsa* I	11 (800)	GGGCCACAATATTTGACCTATATGCACTGGCCCACGCGCCGCAATAGATGAAAATTGCCAAAATTAGCTATCGGTCTTCTGAAAAGAAGGGCCGACATGTTTTCATAGACCATGCAAAGTCATACTACCTGAAACTGATAAATAACGACAAAGAAAGTAGCCTATTTAAAAGTCGCTATAGCATGAATTCCAGCTATGTAGAACTTGATGACCCA...ATTTGTACCTGCCCGGGCCGTCGACCACGCGTGCCCTATAGT
	12 (530)	GGGCCACAATATTTGACCTATATGCACTGGCCCACGCGCCGCAATAGATGAAAATTGCCAAAATTAGCTATCGGTCTTCTGAAAAGAAGGGCCGACATGTTTTCATAGACCATGCAAAGTCATACTACCTGAAACTGATAAATAACGACAAAGAAAGTAGCCTATTTAAAAGTCGCTATAGCATGAATTCCAGCTATGTAGAACTTGATGACCCAGCCATAATTAAGGTGACAGAACAATCTAAAAATGAAAATTTAGAAACTGTTAAGTCACCTGCCCGGGCCGTCGACCACGCGTGCCCTATAGT
LB 426 *Rsa* I	13 (500)	...TTTTTGAAAAAGAAAAAGCCCGAAAGGCGGCAACCTCTCGGGCTTCTGGATTTCCGATCCCCGGAATTAGATCTTGGCAGGATATATTGTGGTGTAACGTTAACAAAGCTTGGGACCGCGGTGGAATTCCAAACGATTCCTTGATGCCTATCTTCGCTATGAT
	14 (300)	TTACTATAGGGCACGCGTGGTCGACGGCCCGGGCAGGATAGCTCTGGATATATTGTGGTGTAACGTTAACAAAGCTTGGGACCGCGGTGGAATTCCAAACGATTCCTTGATGCCTATCTTCGCTATGATTGTATGGTAATAAAGTCTCCACACTCCCGAAAAA
LB 441 *Rsa* I	15 (800)	AGCACGTGAGAGACAAGTAAATCACGAAAGCATTTTTCGGGAGATGTGGAGACTTTATTACCATACAATCATAGCGAAGATAGGCATCAAGGAATCGTTTGGAATTCCACCGCGGTCCCAAGCTTTGTTAACGTTACACCACAATATATCCAGAGCTATCCTTTGGAGCACGTTGAACTCCTAAGATGTTATCTAGACATAAGGTAGAATTTTGGAATTTCTCCAACTTCCTATTT...
LB 441 *Sca* I	16 (300)	GGCCCGGTCTTTTTTTTGAAAAAGAAAAAGCCCGAAAGGCGGCAACCTCTCGGGCTTCTGGATTTCCGATCCCCGGAATTAGATCTTGGCAGGATATATTGTGGTGTAACGTTAACAAAGCTTGGGACCGCGGTGGAATTCCAAACGATTCCTTGATGCCTATCTTCGCTATGATTGTATGGTAATAAAGTCTCCACATCTCCCGAAAAA

Green shade: *rolABC* genes; red: pGreen borders; light blue: pGreen adaptor polylinker; black bold: pGreen backbone; black bold underlined: *Bgl* II restriction site (external to pGreen T-DNA); green underlined: walking primer; blue: adaptor with nested primer (AP2) underlined; black: genomic DNA or sequences of unknown origin.

Miss-recognition of LB and RB

Fragments 1 to 3 and 9 represent cases of backbone integration at the RB. One possible explanation might be the recognition of the LB as initiation site for T-DNA strand with consequent integration of the backbone (van der Graaff *et al*. 1996). Because the RB is not efficiently recognised as T-strand termination, integration continues with the T-DNA (Podevin *et al*. 2006). The other explanation is that integration starts at the RB and continues through the LB with a consequent integration of the entire T-DNA and backbone and an additional copy of the T-DNA (de Buck *et al*. 2000). In both cases, a complete copy of the backbone should be found in the plants of clones 426 and 428. Specific analyses were not carried out for this purpose. The identification in plant 426 of a LB that is read through (13) and of a fragment at RB (2) consisting of a long backbone sequence (1350 bp) support the hypothesis that either a read-through LB or RB may have taken place.

A read-through vector backbone is also evident in fragment 16. The LB junctions 14 and 15 curiously share 11 bases of a putative genomic or filler DNA even though they belong to two diverse plant clones.

Re-arrangements of the T-strand

Recombination of the T-DNA is evident in fragments 1 and 5. Downstream of the RB, the sequence of fragment 1 is characterized by a brief backbone strand between short strands of unknown origin; the sequences continue with a segment of the *rolABC* genes (bases 1204 to 1554). In fragment 5 the *rolABC* genes are nicked 50 bp before the RB; the sequence continues with a *rolABC* sequence from bases 1067 to 1157.

Additional remarks

In most sequenced fragments, the correct adaptor sequence was identified. In one case (fragment 4), the nested adaptor primer follows the T-DNA sequence directly and no restriction site of the *Pvu* II enzyme was found. In additional two cases, the adaptor is ligated to a sequence that does not correspond exactly to the expected restriction enzyme site: fragment 2 (*Pvu* II library: CAC instead of CAG); fragment 12 (*Rsa* I library: one base between the expected GT and adaptor).

AL-PCR analysis (**Table 2**) and sequence of some of the amplified fragments (**Table 3**) demonstrate that none of the three marker-free plant clones expressing *rolABC* integrates a single copy of the genes. In addition, backbone sequences were found in all plants. Taken together these data indicate that this material needs further steps of conventional breeding, such as crosses with non-transgenic plants, to identify progenies that respond to the objective of inserting the minimum amount of DNA required for the correct expression of the transgene(s).

CONCLUSIONS

The absence in transgenic plants of marker genes and further sequences, not essential for the correct expression or silencing of the useful transgene, is a critical requisite to respond either to public concern or to address technical demands. Several papers (Laura *et al*. 2003; Vetten *et al*. 2003; Jia *et al*. 2007) demonstrate that transformation with the useful gene only seems to be the choice procedure for species with a good regeneration ability and transformation competence (e.g. cassava, potato, *Osteospermum* and tobacco). In less easy-to-handle species, the utilisation of a marker gene that gives a selective advantage with mechanisms diverse from resistance to antibiotics/herbicides, the utilisation of visible markers or the elimination of the marker gene from transgenic plants by a range of methods, may be more advantageous. Transformation vectors with a minimum T-DNA characterized by a short polylinker only, such as pGreen adaptor and its derivative (pGreen *rol C* and pGreen *rolABC*), may reduce the extent of unwanted sequences on the T-DNA. In this work, AL-PCR analysis of marker-free plants expressing the *rolABC* and sequencing of derived products confirms previous evidence (Mayerhofer *et al*. 1991; van der Graaff *et al*. 1996), demonstrating that mis-recognition of LB and RB by the *Agrobacterium* machinery is one of the major reasons leading to integration in the plant of the desired genes linked to backbone sequences. Podevin *et al*. (2006) found that the addition of inner and/or outer *Agrobacterium*-derived sequences to the 24 bp repeat of LB enhanced border recognition. The tandem occurrence of octopine and nopaline regions, surrounding the respective LB repeats, gave the highest contribution to the improvement of correct T-DNA transfer (62% correct LB recognition). The effect of tandem copy of LB on correct termination of the T-strand is not clear on the base of the contrasting results of Kuraya *et al*. (2004) and of Podevin *et al*. (2006). Other factors such as *vir* genes carried on resident vector, binary vector and co-cultivation conditions should be taken into account. These factors must be considered during the preparation of further transfer vectors. Rommens (2004) posed a concept of "All native DNA transformation" signifying the opportunity to engineering in a given species genetic material of sexually compatible species only. Concerning this view or in a more realistic view of "cisgenic plants", whose insertion *in planta* is of genetic material from plant only, our aim is to identify plant sequences, similar to the *Agrobacterium* sequence surrounding the LB, that still serve to correctly recognise the LB.

ACKNOWLEDGEMENTS

This work was funded by the Italian Ministry of Agriculture and Forestry. The authors are very grateful to Phill Mullineaux for providing pGreen 0000, pGreenII 0000 and pSoup and for supplying many helpful suggestions.

REFERENCES

An G (1987) Binary Ti vectors for plant transformation and promoter analysis. *Methods in Enzymology* **153**, 292-305

Brunaud V, Balzergue S, Dubreucq B, Aubourg S, Samson F, Chauvin S, Bechtold N, Cruaud C, DeRose R, Pelletier G, Lepiniec L, Caboche M, Lecharny A (2002) T-DNA integration into Arabidopsis genome depends on sequences of pre-insertion sites. *EMBO Reports* **12**, 1152-1157

Cluster PD, O'Dell M, Metzlaff M, Flavell RB (1996) Details of T-DNA structural organization from transgenic *Petunia* population exhibiting co-suppression. *Plant Molecular Biology* **32**, 1197-1203

Cottage A, Yang A, Maunders H, de Lacy RC, Ramsay NA (2001) Identification of DNA sequences flanking T-DNA insertions by PCR-walking. *Plant Molecular Biology Reporter* **19**, 321-327

Dale EC, Ow DW (1991) Gene transfer with subsequent removal of the selection gene from the host genome. *Proceedings of the National Academy of Sciences USA* **88**, 10558-10562

Daley M, Knauf VC, Summerfelt KR, Turner JC (1998) Co-transformation with one *Agrobacterium* containing two binary plasmid as a method for producing marker-free transgenic plants. *Plant Cell Reports* **17**, 489-496

Dalton SJ, Bettany AJE, Timms E, Morris P (1999) Cotrasformed diploid *Lolium perenne* (perennial ryegrass), *Lolium multiflorum* (Italian ryegrass) and *Lolium temulentum* (darnel) plants produced by microprojectile bombardment. *Plant Cell Reports* **18**, 721-726

de Block TD, Debrouwer D (1991) Two T-DNAs co-transformed into *Brassica napus* by a double *Agrobacterium tumefaciens* infection are mainly integrated at the same locus. *Theoretical and Applied Genetic* **82**, 257-263

de Buck S, de Wilde C, van Montagu M, Depicker A (2000) T-DNA vector backbone sequences are frequently integrated into the genome of transgenic plants obtained by *Agrobacterium*-mediated transformation. *Molecular Breeding* **6**, 459-468

Ebinuma H, Sugita K, Matsunaga E, Yamakado M (1997) Selectionof marker-free transgenic plants using the isopentenyl transferase gene. *Proceedings of the National Academy of Sciences USA* **94**, 2117-2121

Gheysen G, Villaroel R, van Montagu M (1991) Illegitimate recombination in plants: A model for T-DNA integration. *Genes and Development* **5**, 287-297

Giovannini A, Zottini M, Morreale G, Spena A, Allavena A (1999) Ornamental traits modification by *rol* genes in *Osteospermum ecklonis* transformed with *Agrobacterium tumefaciens*. *In Vitro Cellular and Developmental Biology – Plant* **35**, 70-75

Gleave AP, Mitra DS, Mudge SR, Morris BAM (1999) Selectable marker free

transgenic plants without sexsual crossing: transient expression of cre recombinase and use of a conditional lethal dominant gene. *Plant Molecular Biology* **40**, 223-235
Goldbrough AP, Lastrella CN, Yoder JI (1993) Transposition mediated repositioning and subsequent elimination of marker genes from transgenic tomato. *Biotechnology* **11**, 1286-1292
Gorbunova VV, Levy (1999) How plants make ends meet: DNA double-strand break repair. *Trend in Plant Science* **4**, 263-269
Hamill JD, Rounsley S, Spencer A, Todd G, Rhodes MJC (1991) The use of polymerase chain reaction in plant transformation studies. *Plant Cell Reports* **10**, 221-224
Hanahan D (1983) Studies on transformation of *Escherichia coli* with plasmids. *Journal of Molecular Biology* **166**, 557
Hellens RP, Edwards EA, Leyland NR, Bean S, Mullineaux MP (2000) pGreen: A versatile and flexible binary Ti vector for *Agrobacterium*-mediated plant transformation. *Plant Molecular Biology* **42**, 819-832
Herman L, Jacobs A, van Montagu M, Depicker A (1990) Plant chromosome/marker gene fusion assay for the study of normal and truncated T-DNA integration events. *Molecular and General Genetics* **224**, 248-256
Kim SR, Lee J, Jun SH, Park S, Kang HG, Kwon S, An G (2003) Trangene structures in T-DNA-inserted rice plants. *Plant Molecular Biology* **52**, 761-773
Komari T, Hiel Y, Saito Y, Murai N, Kumashiro T (1996) Vector carrying two separate T-DNAs for transformation of higher plants mediated by *Agrobacterium tumefaciens* and segregation of transformants free from selection markers. *The Plant Journal* **10**, 165-174
Kumar S, Fladung M (2002) Transgene integration in aspen: Structures of integration sites and mechanism of T-DNA integration. *The Plant Journal* **31**, 543-551
Kuraya Y, Ohta S, Fukuda M, Hiei Y, Murai N, Hamada K, Ueki J, Imaseki H, Komari T (2004) Suppression of transfer of non-T-DNA 'vector backbone' sequences by multiple left borders repeats for transformation of higher plants mediated by *Agrobacterium tumefaciens*. *Molecular Breeding* **14**, 309-320
Jia H, Liao M, Verbelen JP, Vissenberg K (2007) Direct creation of marker-free tobacco plants from agroinfiltrated leaf discs. *Plant Cell Reports* **11**, 1961-1965
Laura M, Morreale G, Berio T, Safaverdi G, Allavena A (2003) Breeding clean transgenic ornamental plants. *Acta Horticulturae* **612**, 121-127
Lazo GR, Stein PA, Ludwig RA (1991) DNA transformation-competent *Arabidopsis* genomic library in *Agrobacterium*. *BioTechnology* **9**, 963-967
Martineau B, Voelker TA, Sanders TA (1994) On defining T-DNA. *The Plant Cell* **6**, 1032-1033
Mayerhofer R, Koncz-Kalman Z, Nawrath C, Bakkeren G, Crameri A, Angelis K, Redei GP, Schell J, Honh B, Koncz C (1991) T-DNA integration: A mode of illegitimate recombination in plants. *The EMBO Journal* **10**, 697-704
McKnight TD, Lillis MT, Simpson RB (1987) Segregation of genes transferred to one plant cell from two separate *Agrobacterium* strains. *Plant Molecular Biology* **8**, 439-445
Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum* **15**, 473-497
Podevin N, de Buck S, de Wilde C, Depicker A (2006) Insight into recognition of the T-DNA border repeats as termination sites for T-strand synthesis by *Agrobacterium tumefaciens*. *Transgenic Research* **15**, 557-571
Rommens C (2004) All-native DNA transformation: A new approach to plant genetic engineering. *Trends in Plant Science* **9**, 457-464
Schmulling T, Schell J, Spena A (1988) Single genes from *Agrobacterium rhizogenes* influence plant development. *The EMBO Journal* **9**, 2621-2629
Spena A, Schmulling T, Koncz C, Schell JS (1987) Independent and synergistic activity of *rol* A, B and C loci in stimulating growth in plants. *The EMBO Journal* **13**, 3891-3899
Stahl R, Hovart H, Van Fleet J, Voetz M, Wetts D, Wolf N (2002) T-DNA integration into the barley genome from single and double cassette vectors. *Proceedings National Academy of Sciences USA* **99**, 2146-2151
Vaira AM, Semeria L, Crespi S, Lisa V, Allavena A, Accotto GP (1995) Resistance to Topoviruses in *Nicotiana benthamiana* transformed with the *N* gene of tomato spotted wilt virus: Correlation between transgene expression and protection in primary transformants. *Molecular Plant-Microbe Interactions* **8**, 66-73
van der Graaff E, den Dulk-Ras A, Hooykaas PJJ (1996) Deviating T-DNA transfer from *Agrobacterium tumefaciens* to plants. *Plant Molecular Biology* **31**, 677-681
Vetten N, Wolters AM, Raemakers K, Meer I, Stege R, Heeres E, Heeres P, Visser R (2003) A transformation method for obtaining marker-free plants of a cross-pollinating and vegetatively propagated crop. *Nature Biotechnology* **21**, 439-442
Zheng S, Henken B, Sofiari E, Jacobsen E, Krens FA, Kik C (2001) Molecular characterization of transgenic shallots (*Allium cepa* L.) by adaptor-ligation PCR (ALPCR) and sequencing of genomic DNA flanking T-DNA borders. *Transgenic Research* **10**, 237-245
Zubko E, Scutt C, Meyer P (2000) Intracromosomal recombination between attP regions as a tool to remove selectable marker genes from tobacco transgenes. *Nature Biotechnology* **18**, 442-445
Zuo JR, Niu QW, Moller SG, Chua NH (2001) Chemical-regulated, site-specific DNA excision in transgenic plants. *Nature Biotechnology* **19**, 157-161