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Generation of phenotypically normal marker-free transgenic plants of *Kalanchoe blossfeldiana* through hairy root induction

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Abstract Multi-Auto-Transformation (MAT) vector system consists of positive selection, using the *ipt* or *rol* gene, with a site-specific recombination and DNA removal system, that generates morphologically normal marker-free transgenic plants without antibiotic selective-agent. This study describes *rol*-type MAT vector (pMAT101) containing *lacZ* gene as a model gene and the removable cassette with *gus* gene in the T-DNA region which was used to produce morphologically normal transgenic Kalanchoe blossfeldiana Poelln. employing *rol* gene as the selectable marker gene and *gus* gene as a reporter gene. Leaf explants inoculated with pMAT101 produced hairy roots with GUS expression on agar-solidified, half-strength MS medium without both plant growth regulators and selective agent under dark condition. These hairy roots produced shoots with Ri syndrome such as dwarfism, wrinkled leaves, and an over abundance of roots as a consequence of the morphogenic action of *rol* gene. They eventually produced morphologically normal shoots without GUS expression on the same fresh MS medium under 16h photoperiod. Molecular analysis of DNA from the hairy roots, shoots with Ri syndrome and morphologically normal shoots revealed that the normal shoots had only *lacZ* gene, and the removable cassette consisting of *rol*, *R* (recombinase) and *gus* genes was excised. This study indicates that the *rol*-type MAT vector could be used for the production of morphologically normal marker-free transgenic *K. blossfeldiana* plants without using selective chemical agents.

Key words: *Kalanchoe blossfeldiana*, MAT vector, removable cassette, *rol* gene, site-specific recombination.

A major focus of plant biotechnology over the last years is the development of improved tools for the genetic modifications of crop plants that offers substantial improvements of agricultural practices, food quality and human health. *Agrobacterium*-mediated transformation and direct gene transformation (electroporation, biolistics) have been widely used for these genetic modifications of crop plants. During transformation, however, only a few plant cells accept the integration of foreign DNA, while most of the cells remain non-transgenic. Therefore, a selectable marker gene is co-delivered with the gene of interest in order to identify and encourage the growth of the rarer transgenic cells. Usually, antibiotic or herbicide-selective agents and their corresponding resistance genes are used for selecting transgenic cells and tissues. Since the selectable marker genes remained in the transgenic plants after transformation have been perceived as an environmental safety issue (Ebinuma et al. 2001; Hohn et al. 2001; Yoder and Goldsbrough, 1994), it is desirable to remove the marker genes prior to the release of the transgenics. Furthermore, in plants where multiple agronomic traits are to be introduced by successive transformation cycles, the multiple selection markers used are also stacked. Moreover, very few marker genes are available for the transformation of plants. Thus, removal of the marker gene would facilitate the use of the same marker gene for multiple rounds of transformation.

Several strategies have been reported in the past for marker gene elimination (reviewed by Darbani et al. 2007; Puchta 2000; Hohn et al. 2001; Miki and McHugh 2004) including co-transformation of T-DNAs followed by segregation of the marker from the trait gene (Daley et al. 1998; Lu et al. 2001), use of transposon-mediated repositioning of the gene of interest and the selectable marker gene (Cotsaftis et al. 2002), homologous recombination between direct repeats (Zubko et al. 2000) and site-specific recombination (Ebinuma et al. 1997). A

Abbreviations: GUS, β-glucuronidase; IAA, indole-3-acetic acid; *ipt*, isopentenyl transferase; MAT, multi-auto-transformation; MS medium, Murashige and Skoog medium; PCR, polymerase chain reaction; *R*, recombinase; Ri, root inducing; *rol*, root loci; RS, recombination site; T-DNA, transfer DNA

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number of site-specific recombinases of prokaryotic and or eukaryotic origin have been shown to function in transgenic plants for such marker elimination studies. Among them multi-auto-transformation (MAT) vector system, developed by Ebinuma et al. (1997), is a unique transformation system using morphological changes caused by oncogene (the *ipt* gene) or rhizogene (the *rol* gene) of *Agrobacterium* as the selection marker.

K. blossfeldiana (2n=34) belongs to the Crassulaceae family having green, succulent perennial with scallop-edged leaves and large umbels of flower clusters held above the foliage. The upright, much-branched growth habit and tolerance of low moisture conditions makes it ideal for indoor plants, groundcover use, rock gardens, raised planters, or containers. Floral colours range from the traditional red to yellow, orange, salmon, to pink and almost any colour in between. There is a substantial interest in the production of this ornamental plant as well as in the continuous development of cultivars with new traits making the plant more attractive for the consumer or contributing to reduce the production costs. Moreover, previous study (Aida and Shibata 2002) and our preliminary experiment show that most of the *Kalanchoe* species including *K. blossfeldiana* have high plant regeneration ability from tissue and cell cultures. Already, transgenic plants with dwarf characters were produced in *K. blossfeldiana* by inoculating a wild type *Agrobacterium rhizogenes* strain (Christensen et al. 2008). Therefore, *Kalanchoe* species can be considered as model plants for conducting biotechnological studies particularly for generation of transgenic plants in ornamental species. In our previous study (Thirukkumaran et al. 2009) we used *ipt*-type MAT vector, pMAT21, for the production of phenotypically normal marker-free transgenic *Kalanchoe blossfeldiana* Poelln.. However, a number of escapes have been found using *ipt* gene as the selection marker. In *rol*-type MAT vector system, on the other hand, the possibility of non-transgenic escapes might be minimized since transgenic tissues could be obtained as hairy roots as a transgenic event. However, successful transformation using *rol*-type MAT vector has been reported only in *Antirrhinum majus* (Cui et al. 2000, 2001) and needs to be evaluated in other plant species.

In this study, we investigated the use of *rol*-type MAT vector, pMAT101 (Figure 1), equipped with the *R/RS* system called 'hit-and-run' cassette for the production of phenotypically normal marker-free transgenic *K. blossfeldiana.* It contains the rol selectable marker, which is responsible for the induction and proliferation of 'hairy roots' by increasing auxin sensitivity, and the R recombinase genes, which are located between two directly oriented RS (recombination site) sequences. The *R/RS* and other site-specific recombination systems mediate the excision of a DNA fragment between two

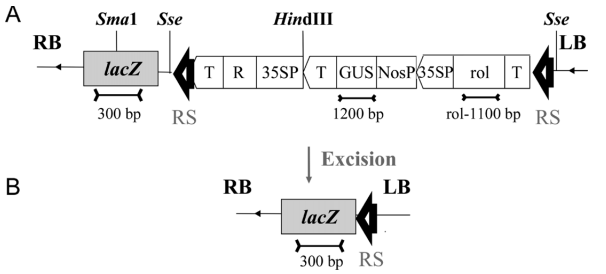

Figure 1. Schematic representation of the T-DNA region of pMAT101. (A) The MAT vector has a "hit-and-run" cassette in which the chimeric *rol* gene is inserted into the R/RS system as a selectable marker. RB, right border sequence of a T-DNA; LB, left border sequence of a T-DNA. The CaMV 35S promoter (35SP) drives the recombinase (*R*) gene. The terminators (T) of *gus*, *rol* and *R* genes are derived from the nopaline synthase. *Gus*, *rol* and *R* genes are located inside the removable cassette flanked by recombination sites (RS). (B) T-DNA region after excision of the "hit-and-run" cassette. Primer positions and length of PCR products are indicated by double arrows. Recognition sites of the restriction enzymes are also indicated.

directly oriented recombination sites in plant cells (Craig 1988; Dale and Ow 1990; Maeser and Kahmann 1991; Onouchi et al. 1991; Russell et al. 1992; Lloyd and Davis 1994). The pMAT101 also contains a-fragment of *lacZ* gene outside of the 'hit-and-run' cassette. The MAT vector system, that includes a recombinase and specific sequences for the enzyme flanking the marker genes and the *rol*, allows elimination of non-useful genes including the *rol* gene after they have fulfilled their role.

Materials and methods

Bacterial strain and vector plasmid

A *rol*-type MAT binary vector plasmid, pMAT101, used in this study was constructed by Sugita et al. (1999), and kindly provided by Nippon Paper Industries, Japan (Figure 1). The pMAT101 plasmid contains a 'hit-and-run' cassette, in which the β-glucuronidase (*gus*) gene under the control of CaMV 35S promoter, the *rol* genes (*rol A, B, C* and *D* genes) of Ri T-DNA and the recombinase gene (*R*) with the 35S promoter are located between directly oriented recombination site (RS) sequences (Ebinuma et al. 1997). A *lacZ* gene is located outside the 'hit-and-run' cassette (Figure 1). The plasmid was transferred from *Escherichia coli* strain DH5α into *A. tumefaciens* strain EHA105 by triparental mating. *A. tumefaciens* strain EHA105 harboring the pMAT101 was used for transformation. *A. tumefaciens* was grown overnight at 28ºC in liquid LB medium (pH 7.2) containing 50 mg l^{-1} kanamycin and 25 mg l^{-1} chloramphenicol. The bacterial suspension was centrifuged (3,000×*g*) for 10 min, decanted the supernatant and re-suspended the bacterial pellet in 1 : 2 (bacterial suspension : MS medium) volume of hormone-free MS medium (Murashige and Skoog 1962) containing 100 μM acetosyringone to give a density of OD_{600}=0.6. The inoculum prepared in this way was used for transformation.

Plant material and transformation

Plants of *Kalanchoe blossfeldiana* cultured under *in vitro*

conditions were used as the experimental material. Newly formed expanding leaves were excised from *in vitro* plants and cut the anterior and posterior ends to make a proper leaf segment (~6–8 mm) for inoculation. According to the method of Horsch et al. (1985), the leaf explants were inoculated by immersing in the bacterial suspension (OD_{600}=0.6) (inoculum) for about 10 min with gentle shaking. They were then blotted dry with sterile filter paper and placed on phytohormone- and selective antibiotic-free half-strength MS medium containing 20 g l^{-1} sucrose, 8 g l^{-1} agar and 100 μM acetosyringone at pH 5.8 for 3 days of co-cultivation under dark conditions. After co-cultivation, the explants were washed with liquid hormone-free MS medium containing 20 mg l^{-1} meropenem (Ogawa and Mii 2004, Meropen; Sumitomo Pharmaceuticals Co., Ltd., Osaka, JPN) without acetosyringone, and transferred to the same medium to control further bacterial growth. Fifty inoculated and twenty-five un-inoculated control explants, having 3 triplicates, were kept under dark conditions for approximately 3 weeks with a weekly subculture on the same MS medium at 25°C.

Root culture and shoot regeneration

Three weeks after inoculation, root tips (approximately 1 cm) of adventitious roots were excised from inoculated explants and transferred to the same fresh MS medium at 25°C under dark condition. Transformed hairy roots identified by their vigorous growth and histochemical GUS assay were cut into 2 cm pieces and cultured on the same MS medium at 25°C under 16 h photoperiod with fluorescent light (35 mmol m^{-2} s^{-1}). These cultures were transferred to the same fresh medium every 2 weeks. Regenerated shoots excised from the hairy roots were cultured on the same fresh MS medium under the same conditions.

Histochemical GUS assay

GUS expression was assayed by soaking the hairy roots and leaves from regenerated shoots, under vacuum (380 mm Hg), in 1 μM X-GLUC (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid, Wako Pure Chemical Industries Ltd, Osaka, Japan) solution (Jefferson 1987) for 15 min, and incubated overnight at 37°C. Chlorophyll was removed by soaking tissues for several hours in 70% ethanol. Tissues stained with indigogenic dye, were scored.

DNA isolation and polymerase chain reaction analysis

Genomic DNAs were extracted from hairy roots and the regenerated shoots, and control *Kalanchoe* plants following CTAB (cetyl trimethyl ammonium bromide) method (Rogers and Bendich 1988). Polymerase chain reaction (PCR) was performed using genomic DNA of each plant as a target and the oligonucleotide primers (Bex Co. Ltd., Japan) for *gus, rol* and *lacZ* genes, as shown in Figure 1.

The PCR products expected to be amplified using these primers were 1200 bp fragment for the *gus* gene, 1100 bp for the *rol* gene, and 300 bp for the *lacZ* gene. DNA amplification was carried out in 20 μl of total volume containing 1.0 μl of 10 ng μl^{-1} plant genomic DNA, 1 μl of 20 pmol μl^{-1} each of the primers, 1.25 μM dNTP (Pharmacia Biotech, Sweden), 2.5 μl of PCR buffer (800 μM KCl, 100 μM Tris-HCl pH 8.3, 15 μM $MgCl_2$, 5 mg ml^{-1} bovine serum albumin, 1% sodium cholate and 1% Triton X-100) and 1 unit of DNA *Taq* Polymerase (Takara Shuzo Co., Japan) per tube. Amplification was carried out in a thermal cycler (PTC-200 Peltier Thermal Cycler) with the optimized cycling parameters for each pair of primers used. After PCR, the amplification products (5 μl) were mixed with loading buffer and run on 1.0% (w/v) agarose (FMC Bio Products, USA) gel at 100 V for 30–40 min with Tris-EDTA as a running buffer. Then the gel was stained with ethidium bromide solution for 20 min, visualized and imaged using Nighthawk™ gel documentation system (PDI Inc., USA). The sequences of the oligonucleotide PCR primers were as follow: *GUS*1, 5′-GGTGGGAAAGCGCGTTACAAG-3′; *GUS*2, 5′-TTTACGCGTTGCTTCCGCCA-3′; *ROL*1, 5′-TGGCTGGC-GGTCTTCGATT CATTTC-3′; *ROL*2, 5′-GTAACTATCCAA-CTCACATCACAAG-3′; *LACZ*1, 5′-GCCAGCTGGCGAAA-GGGGGATGTGC-3′; *LACZ*2, 5′-GAAAGGAAGGGAAGA-AAGCGAAAG-3′.

Results and discussion

Hairy root induction

When leaf explants of *K. blossfeldiana* co-cultivated with *A. tumefaciens* harboring pMAT101 were cultured on plant growth regulator- and selective agent-free agar-solidified medium, adventitious roots were initiated on cut ends of explants one week after co-cultivation under dark condition (Figure 2A). Forty-three out of fifty explants produced adventitious roots, and 149 adventitious roots reached around 1 cm long two weeks after co-cultivation under dark condition. These roots were excised from the explants and cultured on the same fresh MS medium again under dark condition. Twenty-one adventitious roots grew much more vigorously than rest of the adventitious roots (Figure 2B). The former roots displayed a classic hairy root proliferation response -i.e., a set of phenotypic characteristics typical to the roots expressing *rol* genes (Schmulling et al. 1988). Twenty of the 21 vigorously grown adventitious roots and one of the normally grown adventitious roots showed clear GUS expression when all 149 adventitious roots were subjected to histochemical GUS assay (Figure 2C). Moreover, the predicted 1200 bp *gus*, 1100 bp *rol* and 300 bp *lacZ* fragments (Figure 3, lanes 1–3) were amplified using the DNA extracted from twenty-one hairy roots, which were identified by their vigorous growth and GUS assay, with primers GUS1-GUS2, ROL1-ROL2 and LACZ1-LACZ2 respectively. However, these fragments were not amplified in randomly selected ten adventitious roots obtained from uninfected leaf explants. These results indicate that the transgenes were incorporated into the *K. blossfeldiana* genome. The vigorous root growth made it possible to visually select transformed roots, which could be called hairy roots, without using antibiotic selective agent. In contrast, the roots, which failed to show any GUS expression by

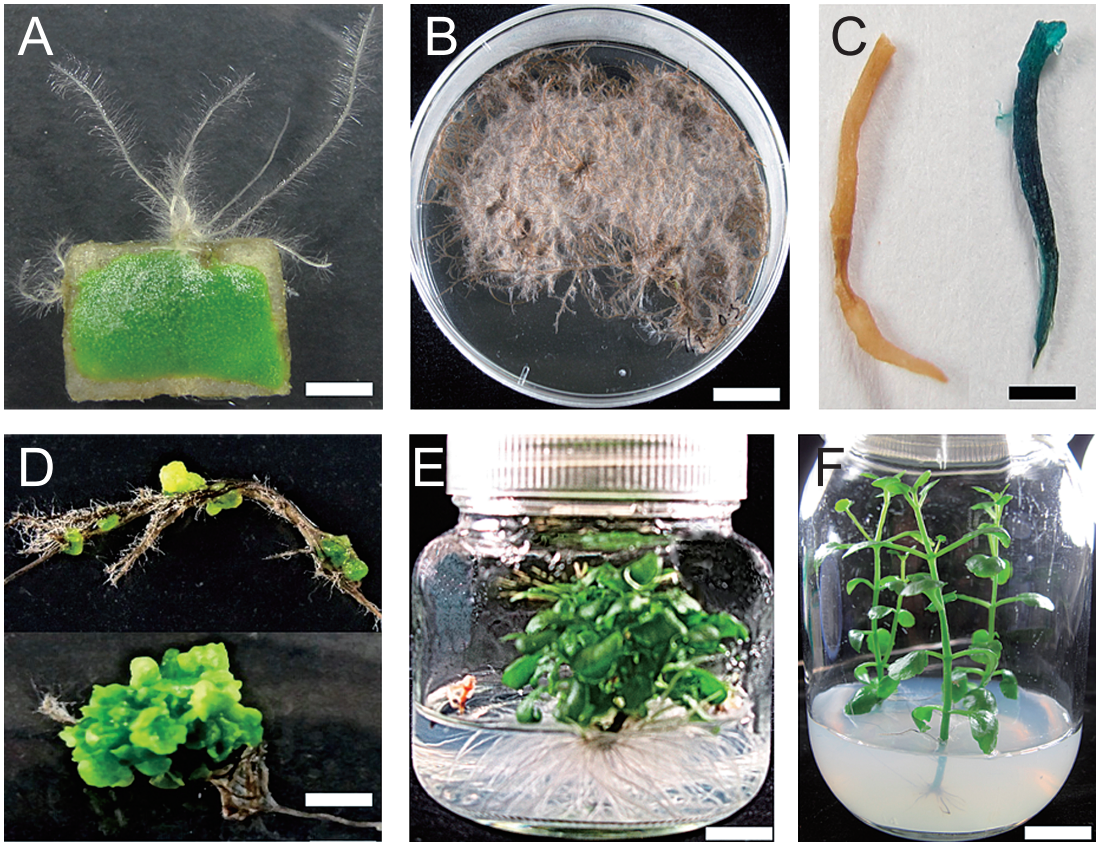

Figure 2. Regeneration of marker-free plants through hairy roots after inoculation of leaf explants with *rol*- type MAT vector. (A) Adventitious root induction, bar=3 mm; (B) Vigorous growth of hairy roots, bar=14 mm; (C) Gus assay of control and hairy root, bar=1 mm; (D) Shoots from hairy roots, bar=3 mm; (E) Abnormal shoots resulting from hairy roots, bar=14 mm; (F) Morphologically normal marker-free shoots, bar=16 mm.

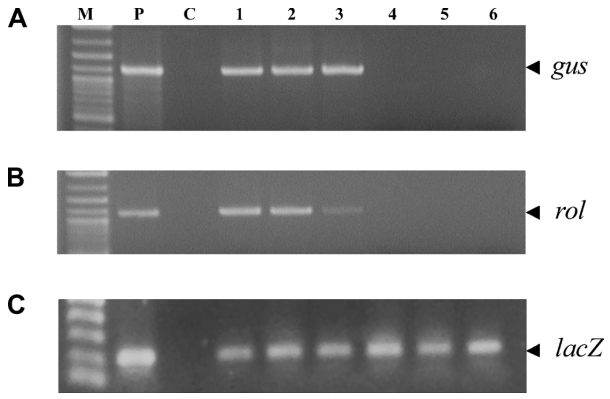

Figure 3. PCR analysis of genomic DNA from hairy roots or abnormal shoots (lane 1–3) and phenotypically normal shoots (lane 4–6). (A) PCR amplification of *gus* (1200 bp) fragment using primers GUS1 and GUS2; (B) PCR amplification of *rol* (1100 bp) fragment using primers ROL1 and ROL2; (C) PCR amplification of *lacZ* fragment (300 bp) using primers LACZ1 and LACZ2. M size markers (Quick-LoadTM 2-Log DNA Ladder), P Positive control from plasmid of MAT vector, C Non-transgenic plant.

histochemical GUS assay, are considered to be non-transgenic escapes produced from the inoculated explants. They might have been caused by endogenous auxin of explant tissue or diffusion of the endogenous auxin from transgenic cells to the adjacent non-transformed cells. It is necessary to clarify the nature of one vigorously grown root that failed to show GUS expression and one GUS expressed root that failed to grow vigorously by conducting further analysis of DNA.

Out of twenty-five un-inoculated leaf explants, eleven explants produced adventitious roots on the same MS medium under dark condition. Since these adventitious roots failed to show vigorous growth and GUS expression after transfer to the fresh medium, they might have been caused by endogenous auxin of explant tissue. Komarnytsky et al. (2004) produced hairy roots from leaf sections of tobacco on hormone-free medium usually 3 weeks post-inoculation, but they routinely added indole-3-acetic acid (IAA) to the cultivation medium to maintain the optimum growth of the transformed roots. In the present study, however, GUS-positive hairy roots, transgenic nature of which was confirmed by histochemical GUS assay and PCR analysis for *gus* and *rol* genes, proliferated well on hormone-free medium (Figure 2C). Moreover, they were maintained as axenic cultures on MS medium like as those of other species such as *Nierembergia scoparia* (Godo et al. 1997) and *Ipomoea trichocarpa* (Otani et al. 1996).

Regeneration of transgenic shoots

GUS-positive hairy roots obtained in the present study produced greenish compact calli (Figure 2D) and eventually shoots (Figure 2E) three months of culture on hormone- and selective agent-free half-strength agar-solidified MS medium containing 20 mg l^{-1} meropenum at 25°C under 16 h photoperiod conditions after co-cultivation. Spontaneous occurrence of shoot regeneration from hairy roots on hormone-free medium has also been reported in some species such as *Antirrhinum majus* (Hoshino and Mii 1998, Cui et al. 2000), *Nierembergia scoparia* (Godo et al. 1997), sweet potato (*Ipomoea batatas*) (Otani et al. 1993) and *I. trichocarpa* (Otani et al. 1996). The shoots regenerated from hairy roots showed Ri syndrome symptoms such as dwarfism, wrinkled leaves, and an over abundance of roots (Figure 2E) as a consequence of the morphogenic action of *rol* genes as reported in other species (Cui et al. 2000; Faiss et al. 1996; Schmülling et al. 1988). Five months after co-cultivation, twelve Ri shoots eventually exhibited phenotypically normal growth (Figure 2F) with no GUS expression when subjected to histochemical GUS assay, suggesting the excision of the *rol* and *gus* genes.

Confirmation of marker-free shoots

All the phenotypically normal shoots, hairy roots and randomly selected shoots with Ri syndrome were subjected to histochemical GUS assay and PCR analysis to confirm transformation and marker excision. As the results, these three gene fragments were amplified in the DNA from randomly selected ten abnormal shoots with Ri syndrome derived from hairy roots. The *rol*-type MAT vector, pMAT101, used in this study contains the *R/RS* fragment called 'hit-and-run' cassette, in which the *gus*, *rol* and *R* (recombinase) genes are located between two directly oriented *RS* (recombination sites) sequences (Figure 1). The site-specific recombination *R/RS* system had been isolated from a circular plasmid, *pSR*1 of *Zygosaccharomyces rouxii* by Araki et al. (1987). This recombination system allows excision of a DNA fragment with an *R* gene product (recombinase) from a plant genome between two directly oriented recombination sites (Onouchi et al. 1991). Consequently, it can be expected in the present study that when the *R*/*RS* system was excised, the 1200 bp *gus* and 1100 bp *rol* fragments were not amplified, and instead the 300 bp *lacZ* fragment was expected to amplify. Actually, expected *lacZ* fragment was found in eleven out of twelve normal shoots appeared from abnormal lines by primers GUS1-GUS2, ROL1-ROL2 and LACZ1-LACZ2, respectively (Figure 3, lanes 4–6). As an exception, one of the normal shoots induced from an abnormal shoot line amplified all three fragments. This may be due to the silencing of the *rol* gene. In our previous study on the production of marker-free transgenic *K. blossfeldiana* plants by using *ipt*-type MAT vector, we observed some non-transgenic normal shoots from *ipt*-shooty line (Thirukkumaran et al. 2009). This may indicate that the non-transgenic shoots were generated from the cells, which obtained excessive cytokinin from the adjacent transgenic cells. In this *rol*-type MAT vector system, all twelve normal shoots, which appeared from independent abnormal shoot lines, were transgenic. Among the twelve normal shoots, eleven shoots were confirmed to be marker-free transgenic since they were *gus*-, *rol*- and lacZ+ by PCR analysis (Figure 3 lanes 4–6).

Hairy root syndrome is a consequence of the morphogenic action of *rol* gene of the Ri plasmid of *A. rhizogens* in infected plants. The cluster of *rol*ABC genes within the 4.3-kb region of the Ri plasmid is sufficient to induce a typical root proliferation response on *A. rhizogenes* infected plants *in vitro* when no exogenous auxins are supplied to the culture medium (Schmulling et al. 1988; Vilaine and Casse-Delbart 1987). These observations provided the foundation for investigating the use of the *rol*ABC cluster as a phenotypic marker for antibiotic resistance gene-free transformation. However, the *rol* genes on the Ri T-DNA co-transformed with the desired genes caused phenotypic abnormalities in the transgenic plants (Hoshino et al. 1998). In tobacco (Hatamoto et al. 1990) and oilseed rape (Boulter et al. 1990), phenotypically normal transgenic plants without *rol* genes were successfully obtained by crossing the co-transformed abnormal plants harboring *rol* genes and *nptII* gene with wild plants as the consequence of segregation, although it required laborious and time consuming processes. In our present

Table 1 Frequency of abnormal and normal shoots derived from hairy roots after inoculation of leaf explants with rol-type MAT vector, pMAT101

Number of explants inoculated	50
Number (%) of explants producing adventitious roots (1 MAC)	43 (86%)
Number of adventitious roots produced	149
Number (%) of adventitious roots growing vigorously (2 MAC)	21 (14.1%)
Number (%) of GUS+ (assay) adventitious roots	20 (13.4%)
Number (%) of *gus*+ and *rol*+ (PCR) adventitious roots (hairy roots)	21 (14.1%)
Number of abnormal shoots (3 MAC)	Numerous
Number (%) of phenotypically normal shoots produced (5 MAC)	12 (8.1%)
Number (%) of *lac*+ (PCR) normal shoots	12 (8.1%)
Number (%) of marker-free transgenic normal shoots (*rol*−, *gus*−, *lacZ*+(PCR))	11 (7.4%)

MAC, months after co-cultivation; GUS+(assay), histochemical GUS assay of roots with blue staining; *gus*+(PCR), PCR amplification of gus gene; *rol*+(PCR), PCR amplification of *rol* gene; *lacZ*+(PCR), PCR amplification of *lacZ* gene; *gus*−(PCR), non-amplification of gus gene by PCR; *rol*−(PCR), non-amplification of *rol* gene by PCR.

study, marker-free transgenic plants were successfully and efficiently obtained by using *rol*-type MAT vector system by removing the *rol* gene from transgenic plants after transformation (Ebinuma et al. 1997).

This study proved that the chimeric *rol* gene can be used as a visible selection marker for *Agrobacterinum*-mediated transformation of *K. blossfeldiana.* Moreover the production of phenotypically normal marker-free transgenic plants is possible without using selective chemical agents when employing *rol*-type MAT vector and it is a promising method to save time and work for the generation of marker-free normal transgenic *K. blossfeldiana* and related species with repeated transformation with various useful genes.

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