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DEVELOPMENT OF NEW TRANSFORMATION METHODS (MAT VECTOR SYSTEM) USING ONCOGENES OF *AGROBACTERIUM* (IPT, ROL ABC)

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INTRODUCTION

Plant transformation is a core technology in the genetic engineering of plants, which is composed of three processes; 1) introduction of genes into plant cells, 2) selection of transgenic cells, 3) regeneration of intact plants. Plant transformation systems using an *Agrobacterium*-infection or a direct gene transfer (particle bombardment, electroporation, etc.) have been developed and widely used to introduce a foreign DNA into plant cells (1). However, the introduced DNA is integrated to plant genome in only a minor fraction of the treated cells during these treatments. It is, therefore, generally essential to select a small number of these transgenic cells from a large excess of non-transgenic cells, and to regenerate intact plants from them. Commonly selective agents (antibiotic, herbicide etc.) and the corresponding resistance genes (selectable marker genes) are used for the selection of transgenic cells (2). In these selection systems, resistant genes are introduced along with desirable genes, and these treated cells containing both non-transgenic and transgenic cells are placed on culture media with selective agents. The non-transgenic cells are killed, while the transgenic cells survive and are selected (negative selection). During this selection process, exogenous plant hormones (auxin, cytokinin, etc.) are added into the media to stimulate regeneration of transgenic plants. However these current transformation systems have three major problems: 1) the selective agents decrease the activity of plant cells to proliferate and differentiate; 2) there is uncertainty regarding the environmental impact of many selectable marker genes; 3) it is difficult to perform repeated transformations in the step-wise process using the same selectable marker to pyramid desirable genes (3-5). In this review, we describe a new plant vector system (called MATVS for multi-auto-transformation vector system) to overcome these difficulties (7-9).

Principle of MATVS

The MATVS is based on a novel principle that morphological changes caused by oncogenes of *Agrobacterium* (*ipt* gene or *rol* genes etc.) can be used for the selection of transgenic and marker-free transgenic plants. In this system, the oncogenes are introduced along with desirable genes, and these treated cells containing both non-transgenic and transgenic cells are placed on a hormone-free culture medium without selective

agents. The transgenic plants are autonomously regenerated and visually selected through endogenous manipulation of plant hormones (positive selection). Usually selectable marker genes have remained in transgenic plants after a transformation. It is, therefore, generally essential that the selectable marker genes induce neither morphological aberrations nor retard growth during the whole life of transgenic plants. Commonly oncogenes have not been used for the selectable marker gene to transform plants because the resulting transgenic plants exhibit a serious abnormal phenotype. The MATVS is designed to remove the oncogenes from transgenic plants after a transformation by inserting them into removal elements (maize transposable element *Ac* (10,11), yeast site-specific recombination system *R*/RS (12,13)) as described below.

Cytokinin-type MAT Vectors

The characteristic feature of MAT vectors (pNPI106 and pNPI132) is the chimerical *ipt* gene fused with a 35S promoter used as a selectable marker. The *ipt* gene codes for the isopentenyl transferase which catalyses a cytokinin synthesis (14,15), and causes proliferation of transgenic cells and differentiation of adventitious shoots (16). Plasmid pNPI106 has a "hit and run" cassette in which the chimerical *ipt* gene is inserted into *Ac* (Fig.1-a) (7). The *Ac* is a maize transposable element that has the ability to move into new locations within a genome (17). In the transposition process, about 10% of the excised *Ac* elements do not reinsert and therefore disappear, or reinsert into a sister chromatid that is subsequently lost by a somatic segregation (18). Therefore we applied the *Ac* to remove the chimerical *ipt* gene from transgenic cells after a transformation. Also pNPI132 has "hit and run" cassette in which the chimerical *ipt* and *R* (recombinase) genes fused with a 35S promoter

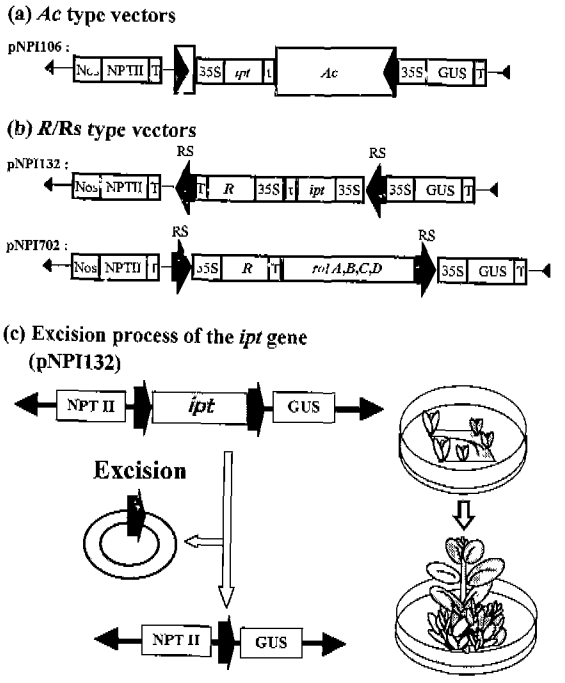

Fig. 1. Schematic diagram of MAT vectors. MAT vectors have a "hit and run" cassette in which the chimeric *ipt* gens or the *rol* gene is inserted into the Ac or the R/RS system as a selectable marker. NPT II and GUS genes are unselected marker genes in these MATVS. These two genes were used as a model of desirable genes. Nos, promoter of the nopaline synthase gene, T, terminator of the nopaline synthase gene, 35S, cauliflower mosaic virus 35S promoter, NPT II, neomycin phosphotransferase gene, GUS, *β*-glucuronidase gene, *ipt*, isopentenyltransferse gene, t, terminator of the *ipt* gene, Ac, maize transposable element, R, recombinase gene, RS, recombination site.

are located between two directly oriented RS (recombination site) sequences (Fig.1-b) (8). The site-specific recombination *R*/RS system had been isolated from a circular plasmid pSR1 of *Zygosaccharomyces rouxii* by Oshima et al., (12). In this recombination system, a DNA fragment between two directly oriented RS is excised from a plant genome with an *R* gene

product (recombinase) (13). Therefore we also applied the excision system of *R*/RS to remove the chimerical *ipt* gene from transgenic cells after a transformation.

Generation of marker-free transgenic plants

We summarize a transformation procedure of MATVS (pNPI106, pNPI132) in tobacco plants (Fig. 2): 1) Tobacco leaf segments were infected with *A .tumefaciens* containing pNPI106 or pNPI132, and cultured on a hormone-free MS medium without a kanamycin (nonselective medium). When adventitious shoots were regenerated (Fig. 2-a), they were separated from the leaf segments and transferred to the same medium; 2) After one month of cultivation, we visually identified and selected abnormal shoots (called ESP for extreme shooty phenotype) (Fig. 2-b) that lost apical dominance for further cultivation; 3) Normal shoots exhibiting normal apical dominance appeared from ESP shoots (Fig. 2-c). We visually identified, selected and transferred these normal shoots to the same medium. These shoots grew normally and rooted (Fig. 2-d). In PCR analysis, the chimerical *ipt* gene was present in the chromosomal DNA of the ESP shoots, but was excised from that of the "normal" shoots along with the "hit and ru n" cassette. These results indicate that these nomally grown shoots are marker-free transgenic plants containing only desired genes.

In the pNPI106 vector, we could obtain marker-free transgenic tobacco plants from only 3 of 63 ESP lines (4.8%) by 8 months after infection with *Agrobacterium* (7). Also we obtained marker-free transgenic tobacco plants from 10 of 48 ESP lines by 4 months after infection in the pNPI132 vector. Finally, marker-free transgenic tobacco plants appeared from 32 of 48 ESP lines (67%) by 8 months after infection (8). These results indicate that the MATVS could produce marker-free transgenic plants without sexual crossings and that the *R*/RS-type MATVS is more practical.

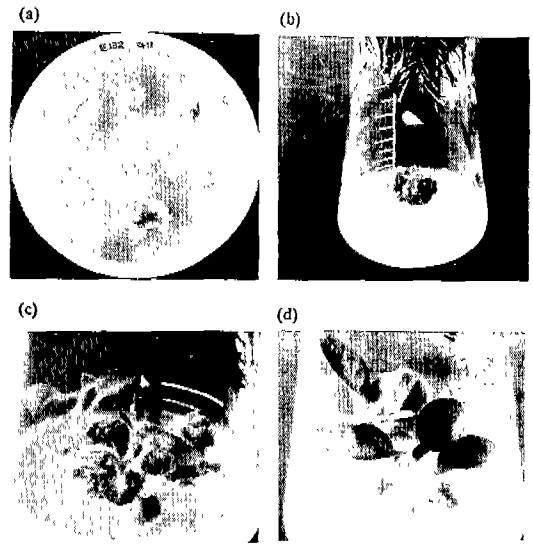

Fig. 2. Thansformation procedure of the MATVS in tobacco plants.
Step 1: Toba leaf segments were infected with A. *tumefaciens* containing MAT vector(pNP I 106 or pNP I 132) and cultured on the hormone-free MS medium without a kanamycin (nonselective medium). Approximately 2-3 weeks later, adventitious shoots were differentiated on leaf segments (a). **Step 2**: After one month of cultivation, independent adventitious shoots were separated and transferred to the same medium After one month, we visually identified and selected abnormal shoots (called ESP;extreme shooty phenotype) that lost apical dominance for further cultivation (b). **Step 3**: Normal shoots the exhibited normal apical dominance appeared from ESP shoots (c). We visually identified, selected and transferred these normal shoots to the same medium These shoots grew and rooted normally (d).

Selection of non-chimerical marker-free transgenic plants

The *ipt* gene fused with a 35S promoter are

reported to increase the cytokinin content up to 137 times in transgenic tobacco shoots (19). These shoots exhibited an ESP that lost apical dominance and rooting ability. We also observed that chimerical shoots comprised with *ipt* transgenic cells and marker(*ipt*)-free transgenic cells exhibited the similar ESP to these cytokinin-overproduced phenotype during the transformation process of the MATVS. Interestingly, we also found a novel phenomenon that non-chimerical marker-free transgenic plants without the *ipt* gene appeared from ESP shoots. We consider that the overproduced cytokinin by the *ipt* gene is released from transgenic cells containing the *ipt* gene to marker-free transgenic cells and maintains an ESP when minor marker-free transgenic cells are coexisting with major *ipt* transgenic cells in ESP shoots. Otherwise, marker-free transgenic cells without the *ipt* gene spontaneously increase in the ESP shoot clones during tissue culture since the *ipt* genes are autonomously dropped from the genome of transgenic cells by removable DNA element of MAT vectors. Consequently, marker-free transgenic shoots were generated from these major fractions of marker-free transgenic cells.

Therefore, we could generate and select non-chimerical marker-free transgenic plants in the MATVS.

Dilemma of negative selectable markers

Recently, transformation methods that utilize the transposable element *Ac*, the site-specific recombination system Cre/*lox* and co-transformation are reported to remove negative selectable marker genes from a transgenic plant (2). (1) *Ac*; The *nptII* gene is inserted into the *Ac* and introduced along with a desirable gene. They moved together with the *Ac* from their integrated loci into another one. As a result, the *Ac* with the *nptII* gene was not genetically linked with a desirable gene. Therefore marker-free transgenic plants were segregated at the second generation by crossings and detected by a PCR analysis. (2) Cre/*lox*; A selectable marker gene (*csr1-1* or *aphIV*) located between two directly oriented *loxP* sequences were introduced along with desirable genes. The *cre* gene for recombinase was introduced along with other selectable marker genes by crossings or second transformation to remove the first introduced selectable marker gene. The second marker gene and first introduced desirable gene were located on different loci. Marker-free transgenic plants were obtained by crossings and detected by PCR analysis. (3) co-transformation; Each the *nptII* gene and a desirable gene were inserted into different binary vector T-DNAs, and co-introduced into different loci of plant genome by an *Agrobacterium*-mediated transformation. The segregated marker-free transgenic plants were detected at the next generation.

In these three methods, both sexual crossings and DNA analysis are essential to segregate the selectable marker gene from a desirable gene, and detect marker-free transgenic plants because three kinds of transgenic cells appear and coexist during the removal process of selectable marker genes in three methods. Three kinds of transgenic cells are ; (1) selectable marker genes are remaining in all copies of inserts, (2) selectable marker genes are eliminating from all copies of inserts (marker-free), (3) several selectable marker genes are eliminating and the other remaining (genomic chimera), However, it is very difficult to select only marker-free transgenic cells using negative selectable marker genes because marker-free transgenic cells are killed on culture media with selective agents.

In contrast to these three methods, the MATVS enables us to select marker-free transgenic plants by morphological changes of transgenic shoots, i.e. the appearance of normal

shoots (marker-free) differed from ESP shoots. These normal shoots were comprised from cells in which the *ipt* gene was eliminated from genome (described above; kind(2)). We are easily, visually and autonomously, able to select marker-free transgenic plants without sexual crossings and DNA analysis using by the MATVS.

Transformation efficiency

The *ipt* genes fused individually to several different promoters were reported to be introduced into potato (20,21), cucumber (22), tobacco (19,23-27), *Arabidopsis* (25), strawberry (28), peach (29) and poplar (30). The cytokinin level of transgenic plants was elevated and the same effect as exogenous applied cytokinin was observed. It is well established that exogenous applied cytokinin can significantly enhance regeneration of shoots from plant tissue cultures in many species. It might be expected that an endogenous manipulation of cytokinin by introduction of the *ipt* gene would work also in a similar way in many other species than tobacco and hybrid aspen. During a negative selection, the majority of the cells in the explants die due to the deleterious effects of selective agents in culture. Such necrotic tissues may release toxic substances, which impair regeneration of transgenic cells, and form a barrier between the medium and the transgenic cells to prevent uptake of essential nutrients. Some plant species may not respond to exogenous applied hormones because of low hormone uptake, compartmentalization, or metabolization. When the *ipt* gene was used as a selectable marker, the explants maintained significantly higher viability during the selection procedure, compared to negative selectable marker genes. Therefore, the MATVS might provide an alternate approach to eliminate such a significant impediment to the recovery of transgenics in recalcitrant plant species, through endogenous manipulation of cytokinin.

Repeated transformation

There are a large number of desirable traits and genes worth incorporating into crop plants. It is possible to introduce a number of genes simultaneously using one selectable marker gene. However it is difficult to modify stepwise a set of related genes such as the genes encoding enzymes of a biosynthesis pathway. Usually sexual crossings are used to pyramid the transgenes into one cultivar of crops. However they cannot be applied to a large number of important crops, including potato, apple, grapevine, strawberries, cassava, banana, as well as hybrids of poplar, and eucalyptus because they are hybrids and must be vegetatively propagated to maintain the elite genome. It is possible to pyramid the transgenes by a repeated transformation, but necessary to use different selectable marker genes for each transformation since the remaining selectable marker genes preclude the same marker gene to introduce a second transgene into the transgenic plants. Also a limited number of selectable marker genes are available for practical use. It is, therefore, desirable to develop a system for generating marker-free transgenic plants, which enables repeated transformation without using up the available marker genes. The previous transformation systems for eliminating selectable marker genes cannot be applied to the hybrid crops because they need sexual crossings to generate marker-free plants and to be able to carry out successive transformation. While the MATVS enables us to generate marker-free transgenic plants without sexual crossings and repeat transformation for pyramiding the transgenes. Moreover, perennial horticultural crops such as fruit trees, and forest tree species such as hybrid aspen, have long reproductive

cycles. A great deal of time would be required to pyramid several valuable genes into trees by conventional breeding. We reported on the removal of a selectable marker gene without crossing from elite clones of hybrid aspen by MATVS (pNPI106) (7). Therefore, MATVS is a most promising way to bypass the difficulties imposed by long generation times and reduces the time required to improve trees through genetic engineering.

Gene inactivation

Usually selectable marker genes are highly expressive in transgenic plant cells for conferring an antibiotic or a herbicide resistance. When we pyramid a number of desirable genes by repeated transformation or crossbreeding of transgenic plants, selectable marker genes are also introduced along with them. It has been reported that the pyramid of homologous DNA sequences results in deleterious interference in gene expression (31). Such a transgene inactivation is a potential problem for commercialization of transgenic crop plants. To avoid this problem, the transgenes should be driven by different promoters and should not be linked to the same selectable marker. However there are only a limited number of promoters and selectable markers available for practical use. It is desirable, therefore, to develop a system for the generation of marker-free transgenic plants to pyramid a number of transgenes in transgenic crop plants.

Auxin type MAT Vectors

The *rol* genes are used as a selectable marker in MAT vector (pNPI702) (9). The *rolA*, *B*, and *C* genes are responsible for proliferation of roots (hairy roots) by increasing auxin sensitivity. Transgenic plants regenerated from hairy roots display abnormal phenotype such as wrinkled leaves, reduced apical dominance or shortened internodes (32). Plasmid pNPI702 has a "hit and run" cassette in which the 7.6-kb DNA fragments containing *rolA*, *B*, *C*, and *D* genes and *R* genes with a 35S promoter are located between two directly oriented RS

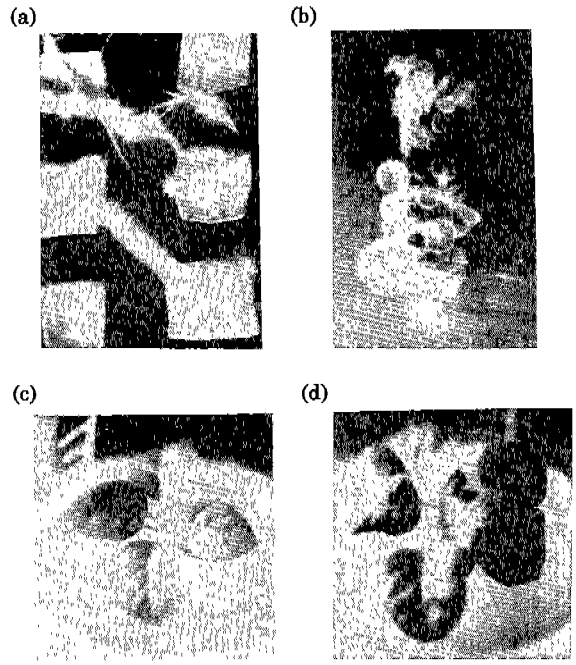

Fig. 3. Transformation procedure of the *rol*-type MATVS in tobacco plants.
Step 1:Tobacco leaf segments were infected with A. *tumefaciens* containing MAT vector(pNP1702) and cultured on the hormone-free MS medium without a kanamycin(nonselective medium). Approximately 2-3 weeks later, hairy roots were differentiated on leaf segments (a). **Step 2**: After 6 weeks of cultivation, independent hairy roots were separated and transferred to the shoot inducing medium. After two months, a lot of shoots were regenerated from hairy roots (b). **Step 3**: These independent shoots were separated and transferred to the hormone-free MS medium. These regenerated shoots resulted in a normal (c) or abnormal (wrinkled-leaf) shoots (d).

sequences (Fig. 1-c). We summarize a transformation procedure of MATVS (pNPI702) in tobacco plants (Fig. 3): 1) Tobacco leaf segments were infected with *A. tumefaciens* containing MAT vector (pNPI702) and cultured on a hormone-free MS medium without kanamycin (nonselective medium). Hairy roots were regenerated two weeks after infection (Fig. 3-a). After one month, they were separated from the leaf segments and transferred to a shoot inducing medium with hormones; 2) After two months half of cultivation, transgenic shoots were regenerated from such roots (Fig. 3-b). We separated these shoots and transferred to a nonselective medium for further cultivation. 3) We visually identified and selected normal plants (Fig. 3-c) from abnormal plants with wrinkled leaves, reduced apical dominance or shortened internodes (Fig. 3-d). We obtained 55 normal ones of 175 plants which were regenerated from 13 roots, and subjected them to a PCR analysis. As a result, only 6 plants from 2 hairy root lines were marker-free transgenic plants and the other 49 plants were either non-transgenic plants, transgenic plants with *rol* genes or chimerical plants. These results indicate that it is difficult to select visually non-chimerical marker-free transgenic plants using the *rol* genes at the R_0 generation. Commonly, crossing of chimerical plants segregates non-chimerical progenies out. Therefore, we subjected the seedlings of chimerical plants to PCR analysis and observed the segregation of non-chimerical marker-free transgenic plants with a high frequency. These results indicate that the MATVS (pNPI702) also could generate non-chimerical marker-free transgenic without sexual crossings, and produce ones more efficiently through crossings.

Advantage of the *rol* genes

In contrast to the *ipt* gene, a number of works were reported to transform plants using the *rol* genes and regenerate transgenic plants. *A. rhizogenes* are susceptible to a wide variety of dicotyledonous plants (116 species) (33). The transgenic plants have been regenerated from hairy roots of 53 plant species (34). Therefore *A. rhizogenes* has been used to transform many recalcitrant plant species, including fruit trees and forest trees. Currently, it was reported that transgenic plants were regenerated from the hairy roots by an infection of *A. rhizogenes* containing both binary vector T-DNA with a desirable gene and the Ri T-DNA. Half of these transgenic plants had desirable genes in addition to the Ri T-DNA. However the characteristic altered phenotype caused by the integrated Ri T-DNA limits their use for commercial genetic engineering. Therefore these transgenic plants were crossed to segregate the insertion of binary vector T-DNA from that of Ri T-DNA. Penotypically normal transgenic progenies were obtained in tobacco (35) and oilseed rape (36). However the segregation frequency of these marker-free transgenic plants appears to be much lower than that obtained by MATVS. Because usually multiple copies of both binary vector T-DNA and Ri T-DNA were inserted into the genome of transgenic plants, most of binary vector T-DNAs were not able to segregate from independent each Ri T-DNAs at meiosis. These results indicate that the *rol* genes have the advantage of efficient transformation for recalcitrant plant species and the MATVS enables us to overcome their disadvantage of altered phenotype.

Application of MATVS to hybrid aspen

We infected *A. tumefaciens* containing pBI121 vectors in which the chimeric *ipt* gene with 35S promoters is inserted (pIPT5) into 20 stem segments from hybrid aspens and transferred them to nonselective medium. One month later, adventitious shoots were

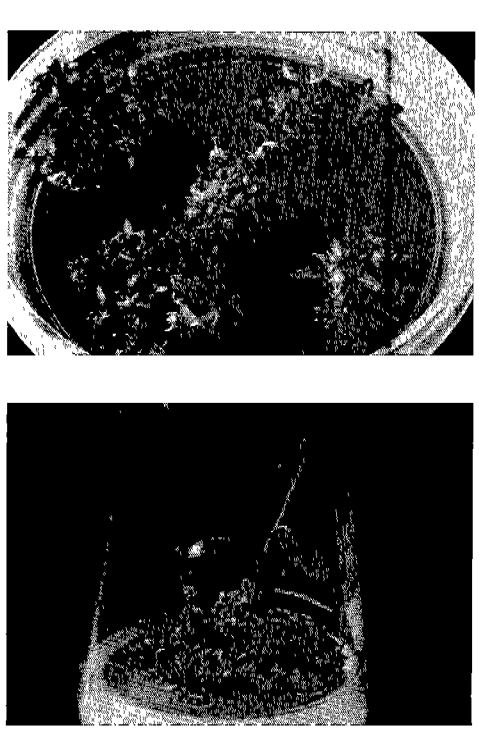

Fig. 4. Transformation of hybrid aspens by pNP I 106.
a) Differentiation of the extreme shooty phenotype (ESP) from adventitious shoots,
b) Reappearance of "normal" morphological shoots from ESP.

differentiated on the stem segments. Two months after infection, we visually identified 20 ESP shoots and 77 normal shoots which were separated for further cultivation to confirm morphology. As a result, we could obtain transgenic shoots from 14 of 25 stem segments (56%). While in current transformation methods, we placed infected stem segments with *A. tumefaciens* containing pBI121 on selective medium with Kanamycin and hormone. Four months after infection, we could only observe shoot formation on 25% of the infected stem segments. These results show that selection systems using the *ipt* gene could shorten the time for shoot formation and increase transformation efficiency.

We inoculated *A. tumefaciens* containing pNPI106 into 50 stem segments from hybrid aspens (7). These infected stem segments were transferred to nonselective medium. After cultivation for one month, adventitious shoots appeared and were transferred to fresh medium. We separated 20 ESP shoots and cultured them (Fig. 4-a). Normal shoots appeared in three ESP shoot clones by 8 months after infection (Fig. 4-b). We separated the normal shoots and transferred them to root inducing medium (2/3 MS medium, 2% sucrose, 0.25% Gelrite, 0.05 mg/l IBA). These shoots grew normally and rooted. By PCR analysis, the *ipt* gene is detected in 20 ESP shoots but not 6 "normal shoots" (2 x 3 lines). These results demonstrate that marker free plants can be selected visibly using a chimeric *ipt* gene in hybrid aspens also.

Improvement of MATVS for practical use

In pNPI132 vectors, three 35S promoters are used to control GUS, ipt and R genes. We found the following problems during transformation process of pNPI132. (1) Excision of the *ipt* genes in *A. tumefaciens*: We applied both pNPI106 and pNPI132 vectors for transformation of hybrid aspens but could not get any ESP shoots using pNPI132. As *A. tumefaciens* strains containing pNPI132 vectors was maintained for several times, the ESP formation efficiency was decreasing dramatically. The recombinase driven by 35S promoters were constitutively expressing in *A. tumefacien* and removing the *ipt* genes before ESP formation. (2) GUS gene silencing: We detected the integration of GUS genes in the isolated ESP shoots by PCR analysis but could not observe the GUS activity in some shoots because of gene silencing. Therefore, we constructed new practical vectors to improve these difficulties (Fig. 5). They have either NPT

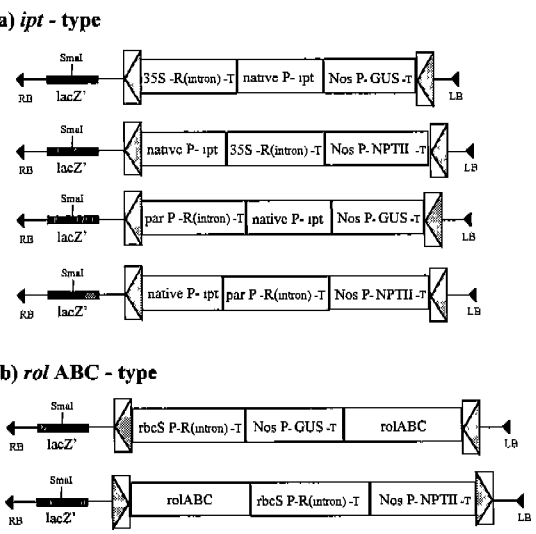

Fig. 5. Schematic diagram of practical MAT vectors

Practical Mat vectors have either NPT II or GUS genes as a second selectable marker in "hit and run" cassette. The chimeric R genes with inducible promoters (par, rbcS) have intron sequences of histon genes not to work in A. *tumefaciens*. The Sma I sites of lacZ′ genes are available for cloning of desirable genes. Native-P, own promoters of *ipt* genes; ParP, par promoters; rbcSP, rubsco promoters; intron, inron sequences of histon genes(Eucalypts).

II or GUS genes as a second selectable marker in "hit and run cassette" to identify the transgenic shoots and roots correctly. The *R* genes of this cassette are inserted with the intron sequences of histon genes and fused with inducible promoters to regulate the activity of recombinase before ESP formation.

Conclusion remarks

We have learned a plant transformation method from phytopathogenic bacteria (*A. tumefaciens*, *A. rhizogenes*). They have a wide host range, and are able to induce "crown gall s" or "hairy roots" in most dicotyledonous and some monocotyledonous plants, and various Gymonosperms. Crown galls and hairy roots result from the expression of oncogenes on T-DNA, which is transferred and integrated into the plant genome during infection. These oncogenes modify the plant hormonal level and balance, or the hormone signal perception of the cells to proliferate tumorous tissues. However, endogenous hormonal levels and cell responses to plant hormones are very different in plant species and plant tissues, and at developmental stages. Therefore, they highly regulate their expression and manipulate auxin/cytokinin ratio according to the physiological and developmental state of plant tissues to achieve wide adaptability.

In current methods, we have utilized only the gene transfer system of *Agrobacteria* but not a plant hormone regulation system of oncogenes for transformation. Alternatively we have applied exogenous plant hormones to proliferate and regenerate transgenic tissues. However, such an approach has inherent difficulties associated with penetration, transport and degradation of plant hormones in tissues, and can not achieve a fine regulation of the hormonal balance. In contrast, oncogenes appear to have higher flexibility for regulation of plant hormones by endogenous manipulation. However oncogenes have not been commonly used for transformation because the resulting transgenic plants show abnormal morphology. In this review, we indicate that a plant hormone regulation system of oncogenes can be used for a plant transformation by combining with removal elements. The flexibility of oncogenes system enables us to improve transformation efficiency in a wide variety of plant species using MATVS.

Additional note

The evaluation works of MATVS (pNPI 106, 132, 702) summarized in this review are in

the application process for a international patent (WO 96/15252). The plasmid materials and manuals of MATVS have been released for researchers through the MAT Vector Association (President: Atsushi Komamine).

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