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# Obtaining marker-free transgenic soybean plants with optimal frequency by constructing a three T-DNA binary vector

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**Abstract** Obtaining marker-free plants with high efficiency will benefit the environmental release of transgenic crops. To achieve this point, a binary vector with three T-DNAs was constructed using several mediate plasmids, in which one copy of *BAR* gene expression cassette and two copies of *VIPI* gene expression cassette were included. EHA101 *Agrobacterium* strain harboring the final construct was applied to transform soybean cotyledon nodes. Through 2–3 months regeneration and selection with 3–5 mg·L<sup>-1</sup> glufosinate, transgenic soybean plants were obtained at 0.83%–3.16%, and the co-transformation efficiency of both genes in the same individual reached up to 86.4%, based on the southern blot test. Using PCR analysis, southern blot and northern blot tests, as well as leaf painting of herbicide in T<sub>1</sub> progenies, 41 plants were eliminated of *BAR* gene with the frequency of 7.6%. Among the T<sub>1</sub> populations tested, the loss of the alien genes happened in 22.7% lines, the silence of the *BAR* gene took place in 27.3% lines, and *VIPI* gene silence existed in 37.1% marker-free plants. The results also suggested that the plasmid with three T-DNAs might be an ideal vector to generate marker-free genetically modified organisms.

**Keywords** *Glycine max*, *Agrobacterium tumefaciens*, transformation, marker-free plants, three T-DNA vector

## 1 Introduction

Breeding by genetic engineering has been regarded as a potential way to improve crop yield, quality, resistance and tolerance since the beginning of the 20th century. In fact, some genetically modified organisms have been successfully applied in practice on a large scale, such as

transgenic soybean, cotton, maize, and rapeseed (Crawley et al., 2001; James, 2002; Keller and Carabias, 2001). More transgenic plants have been developed in recent years (Conner et al., 2003). However, most transgenic organisms cannot be used in yield production because of the bio-safety obstacle, which has been emphasized by many countries in recent years. Selecting marker gene is one of the biggest concerns in bio-safety. There are several strategies to exclude the selection gene for marker-free plants in transgenic generations, such as co-transformation (Depicker et al., 1985; McKnight et al., 1987; De Block and Debrouwer, 1991), site-specific recombination (Dale and Ow, 1991; Gleave et al., 1999), multi-auto-transformation vector (Ebinuma et al., 1997), transposition system (Goldsbrough et al., 1993) and homologous recombination (John et al., 1994), among which co-transformation has been widely used. Particle gun can deliver a mixture DNA of two plasmids carrying a target gene and a selection gene into plant cells, but the obtaining efficiency of marker-free plants is very low in T<sub>1</sub> or T<sub>2</sub> progeny (Yohichi et al., 1998). Two *Agrobacterium* strains implementing two binary vectors (Depicker et al., 1985; McKnight et al., 1987; De Block and Debrouwer, 1991) and one *Agrobacterium* strain harboring two binary vectors (Daley et al., 1998) or one binary vector with two T-DNAs containing target gene and selection gene (Depicker et al., 1985; Komari et al., 1996; Xing et al., 2000; Shirley et al., 2004) can also be used to get marker-free plants, but the efficiency of the marker-free plants was also very low. In this research, we constructed a binary vector with three copies of T-DNA, one for the selection gene and two for the target gene, from which a lot of marker-free plants were obtained with high efficiency.

## 2 Materials and methods

### 2.1 Vectors and bacteria strains

Several cloning vectors and expression vectors were used in this study to construct a multiple T-DNA binary vector

Translated from *Chinese Journal of Biotechnology*, 2007, 23(1): 138–144 [译自: 生物工程学报]

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(with *VIP1* as the gene of interest and *BAR* as the selection gene) for obtaining marker-free transgenic plants, such as Topo, pRTL2, pZP100 and pPTN200.

Bacterial strain DH5 $\alpha$  and *Agrobacterium* strain EHA101 (Hood et al., 1986) were used to propagate the construct and to transform soybean, respectively.

## 2.2 Soybean material and transformation

A commercial soybean variety, Thorne, was used in this study for stable transformation due to its good regeneration ability and susceptibility to infection by *Agrobacterium*. Mature seeds were surface-sterilized twice with chlorine gas (produced from the fresh mixture of 100 mL bleach and 3.3 mL 12 mol·L<sup>-1</sup> HCl) in a sealed container for about 24 hours each time. The sterilized seeds were then put on B5 basal medium for 5 days of germination under light condition.

The *Agrobacterium*-mediated soybean cotyledonary-node transformation system described by Hinchey et al. (1988) was applied, in which glufosinate was used as a selection agent to screen transgenic plants (Zhang et al., 1999; Clemente et al., 2000; Xing et al., 2000; Shirley et al., 2004). After 3 days of co-cultivation between soybean cotyledonary nodes and *Agrobacterium*, the explants were cultured on B5 medium with 1.67 mg·L<sup>-1</sup> 6-BA and 5.0 mg·L<sup>-1</sup> glufosinate for 4 weeks to induce multiple resistant shoots, and then sub-cultured on MS medium with 1.0 mg·L<sup>-1</sup> ZA, 0.5 mg·L<sup>-1</sup> GA<sub>3</sub>, 0.1 mg·L<sup>-1</sup> IAA, and 3.0 mg·L<sup>-1</sup> glufosinate to elongate the shoots.

## 2.3 Molecular tests

Genomic DNA and total RNA were extracted from the leaves of T<sub>0</sub> plants and T<sub>1</sub> progenies as described by Dellaporta et al. (1983) and Sambrook et al. (1989), respectively. Molecular tests of PCR, southern blot and northern blot were then applied to identify positive plants and check the target gene transcriptions. The primers for the *VIP1* gene are 5'-TCATGAGTGTGATTTCGGAAGA-AACC-3' and 5'-TCTAGATCAGCCTCT CTGG-TGAAAT-3'. The primers for the *BAR* gene are 5'-AAGCACGGTCAACTTCCGTA-3' and 5'-GAAGT-CCAGCTGCCAGAAAC-3'. The *VIP1* gene and *BAR* gene isolated from related vectors were used as probes to be hybridized with genomic RNA and genomic DNA, which was digested with *SstI* for 16–17 hours.

## 2.4 Leaf painting

A 0.1% solution of Liberty was daubed onto the younger leaves of T<sub>0</sub> regeneration plants and T<sub>1</sub> individuals with a cotton swab to test their herbicide tolerance. The symptoms caused by the chemical were observed 3 to 5 days after application.

## 3 Results

### 3.1 Plasmid construction of multiple T-DNAs

The cDNA of the *VIP1* gene (783 bp) was introduced into the Topo vector (3.9 kb) as the first step for cloning and enzyme site incorporation, and then the extended *VIP1* piece (810 bp) was cut with *EcoRI* from the recombination vector to be integrated into another vector pRTL2 (3.9 kb) in order to get the 35S promoter, TEV enhancer and polyA terminator. Then, the *VIP1* expression cassette (1.9 kb) was isolated by *PstI* from the new construct and inserted into a plant binary vector pZP100 (6.6 kb) between the right border (RB) and the left border (LB). Afterwards, the T-DNA fragment with *VIP1* expressing elements (3.4 kb) was cut from the previous plasmid with *ScaI* and introduced into a second plant binary vector pPTN200 (8.7 kb), in which the *BAR* gene expression cassette was included in a different T-DNA region with the *NOS* promoter, *BAR* ORF and *NOS* terminator. At last, 12 clones were obtained from a ligation. Digested by *PstI*, one clone showed four right bands of 0.6 kb, 1.9 kb, 4.5 kb, and 8.5 kb. The putative clone was further confirmed by additional restriction enzyme digestion, and different bands were produced from different enzymes: 0.5 kb, 5.5 kb, and 8.5 kb from *HindIII*; 2.5 kb, 3.9 kb, and 9.1 kb from *BamHI*; 3.4 kb and 8.7 kb from *ScaI*; 2.5 kb, 3.9 kb, and 9.1 kb from *SstI*; 3.8 kb and 11.7 kb from *EcoRV*; and 0.8 kb, 2.5 kb, 3.4 kb, and 8.8 kb from *EcoRI*. The results indicated that a binary vector with three T-DNA, one for *BAR* gene and two for *VIP1* gene, was successfully constructed, referred to as pPTN363 (Fig. 1).

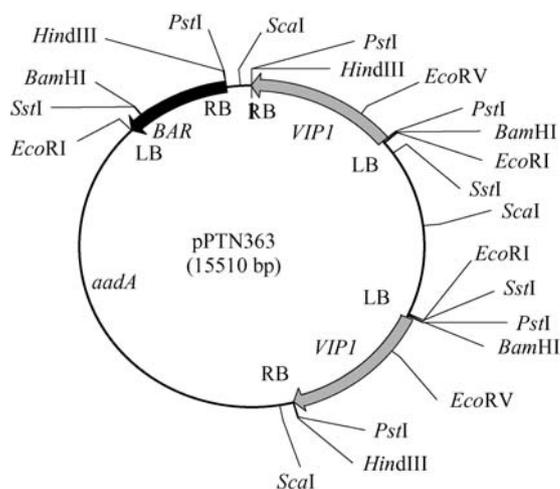


Fig. 1 Assembly of the three T-DNA binary vector

### 3.2 Transformation and identification of T<sub>0</sub> plants

The new binary vector, pPTN363, was transformed into EHA101 by triparental mating, and then used to infect

soybean explants. Twelve transformation experiments were set up, and 59 plants resistant to both glufosinate on medium and Liberty on leaves were finally obtained. By southern blot analysis probed with *VIPI* gene, 51 herbicide-resistant plants were confirmed to be positive with 1–9 integration copies in different individuals, with the transformation efficiency ranging from 0.83% to 3.16%. The hybridized bands varied from 2.7 kb to 19.0 kb (Fig. 2, Table 1). The results also indicated the co-transformation efficiency of both genes was 86.4%. Northern blot analysis indicated that DNA transcription was evident in 24 positive plants, which showed a 2.0 kb hybridized band, and gene silencing occurred in the 27 other positive plants, representing 52.9% of all positive plants (Fig. 3).

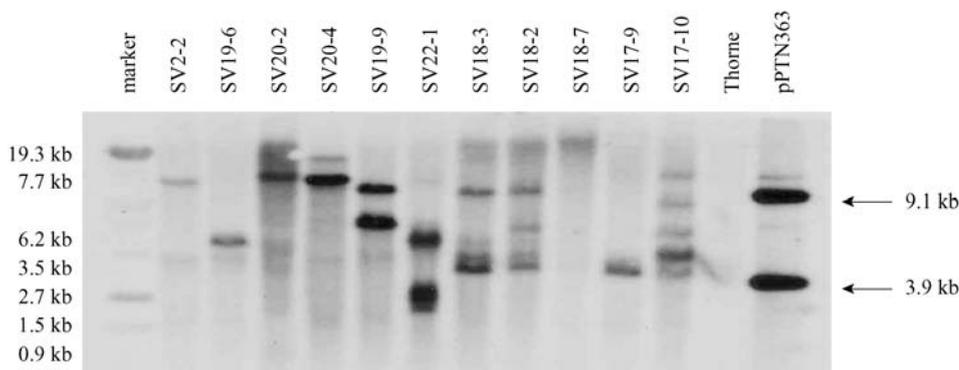
### 3.3 Screening of marker-free transgenic plants in T<sub>1</sub> progenies

The T<sub>1</sub> progeny was screened by leaf painting of the herbicide for *BAR* gene first (Fig. 4), and herbicide-sensitive plants were tested by PCR for *VIPI* gene next (Fig. 5). Then, the possible marker-free and *VIPI* positive plants

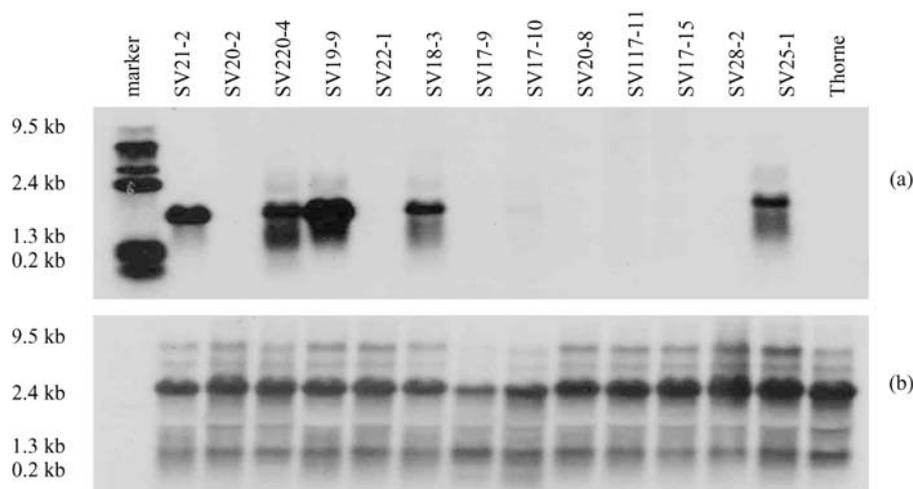
**Table 1** Transgenic soybean plants obtained from each experiment

| experiment | explants cultured | positive plants obtained | transformation frequency/% |
|------------|-------------------|--------------------------|----------------------------|
| SV17       | 285               | 9                        | 3.16                       |
| SV18       | 250               | 5                        | 2.00                       |
| SV19       | 270               | 7                        | 2.59                       |
| SV20       | 300               | 6                        | 2.00                       |
| SV21       | 245               | 4                        | 1.63                       |
| SV22       | 285               | 3                        | 1.01                       |
| SV23       | 240               | 2                        | 0.83                       |
| SV24       | 250               | 3                        | 1.20                       |
| SV25       | 280               | 4                        | 1.43                       |
| SV26       | 140               | 2                        | 1.43                       |
| SV27       | 100               | 2                        | 2.00                       |
| SV28       | 200               | 4                        | 2.00                       |
| total      | 2845              | 51                       | 1.79                       |

were confirmed further by both PCR analysis for the two genes in one reaction and southern blot tests probed with *VIPI* and *BAR* genes (Figs. 6–7). Five hundred and thirty nine T<sub>1</sub> plants from 22 independent events were analyzed in total (Table 2), and 41 marker-free plants (*VIPI*<sup>+</sup>*BAR*<sup>-</sup>), such as V7-6, V7-10, V7-13, V7-16



**Fig. 2** Southern blot analysis of putative primary T<sub>0</sub> transformants  
Note: pPTN363 represents positive control; Thorne represents negative control.



**Fig. 3** Northern blot analysis of putative primary T<sub>0</sub> transformants  
Note: (a): probed with *VIPI* gene; (b): probed with rDNA



**Fig. 4** Possible marker-free plants screened out in T<sub>1</sub> progenies by leaf painting

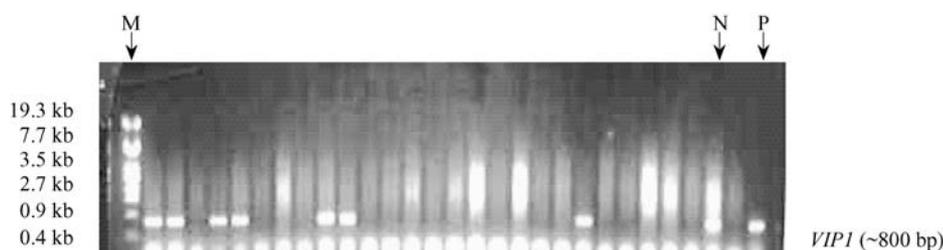
Note: The herbicide sensitive plant, indicated by the arrow, was marker-free or empty of the alien gene.

(Fig. 7), were identified by an average frequency of 7.6%, among which 20 marker-free plants originated from the SV19-17 line, 10 marker-free plants from the SV20-2 line, 5 marker-free plants from the SV20-4 line, 4 marker-free plants from the SV20-8 line, one marker-free plant from the SV17-11 line, and one marker-free plant from the SV18-2 line. At the same time, 82 plants with the *BAR* gene but without the *VIPI* gene (*VIPI*<sup>-</sup>*BAR*<sup>+</sup>), 149 plants with both genes (*VIPI*<sup>+</sup>*BAR*<sup>+</sup>), and 267 plants with neither *BAR* gene nor *VIPI* gene (*VIPI*<sup>-</sup>*BAR*<sup>-</sup>) were also identified. These results indicated that the separation of both genes happened in T<sub>1</sub> populations at 22.6%, the loss of both genes in the progenies at 49.5%, and the link inheritance of both genes in T<sub>1</sub> populations at 27.6%. Moreover, all tested plants in SV21-2, SV18-3, SV25-1, and SV28-2, and most tested plants in SV19-6, SV21-1,

SV18-2, and SV22-1 proved to be negative. Closely linked inheritance of both foreign genes was found in most plants of SV17-14, SV18-10, SV17-2, SV20-4, SV18-1, SV19-9, SV20-2, and SV20-8. In addition, the positive plants identified in SV17-1, SV17-5, SV19-6, SV21-1, SV17-9, and SV22-1 contained only the selection gene, and did not contain the gene of interest.

## 4 Discussion

The *VIPI* gene in the recombination vector pPTN363 originated from *Arabidopsis*, which consists of 783 nucleotides. It was found that the *VIPI* protein can be combined with the VirE2-T-DNA complex and elevate T-DNA nuclear import from the cytoplasm and its integration into host chromosome. In a previous study, *VIPI*-antisense transgenic tobacco plants exhibited reduced gall formation following infection with wild-type *Agrobacterium* as compared with the control. The reduced susceptibility was correlated with the reduction of *VIPI* transcript in native tobacco, as confirmed by RT-PCR. Transgenic tobacco with *VIPI* showed more susceptibility to *Agrobacterium* infection, stronger *GUS* gene transient expression, and higher stable transformation frequency (Tzfira et al., 2001, 2002). *Agrobacterium*-mediated transformation efficiency in soybean could be improved by transferring *VIPI* into the soybean and developing a susceptible transgenic material without selection marker. Based on such a purpose, a binary vector with three T-DNAs was constructed for marker-free plants in this study. As a result, plants with the *VIPI* target gene but without the *BAR* selection marker (*VIPI*<sup>+</sup>*BAR*<sup>-</sup>) were obtained at 7.6%, on average. For some independents, the frequency was higher than 14.3%, such



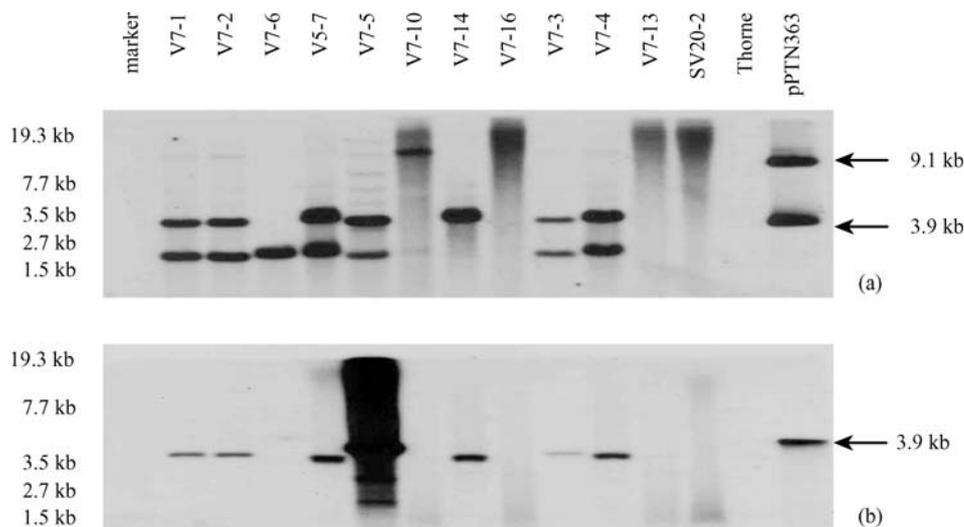
**Fig. 5** PCR analysis of herbicide sensitive T<sub>1</sub> plants for *VIPI* gene

Note: M, N and P represent marker, negative control and positive control, respectively.



**Fig. 6** PCR analysis of herbicide sensitive and *VIPI* positive T<sub>1</sub> plants for both genes

Note: M, N and P represent marker, negative control and positive control, respectively.



**Fig. 7** Southern blot analysis of putative primary T<sub>1</sub> marker-free plants  
 Note: A represents probed with *VIP1* gene and B represents probed with *BAR* gene.

as 22.2% for SV20-8, 34.5% for SV20-2 and 71.4% for SV19-17. In six of the 22 independent T<sub>1</sub> lines tested, marker-free plants were generated, accounting for 27.3%, with both inherited genes linked closely in eight lines, representing 36.4%, and both genes completely lost in six lines, representing 27.3%. Moreover, the separation of both genes happened in fifteen lines at 68.2%. The above results demonstrated that the recombination plasmid with three T-DNA might be an ideal binary vector for use in *Agrobacterium* mediated transformation to get marker-free plants by initiating the segregation of the target gene and the selection gene in T<sub>1</sub> generation. The marker-free plants

obtained in this research were self-pollinated for *VIP1* homozygotes, and then used to re-transform other function genes to check the role of *VIP1* in soybean. Future experiments and results will be reported later in other papers.

Except for T-DNA being lost at a higher rate from the transgenic soybean plants, gene silencing was also regularly found in this research. The *VIP1* gene was silenced in 52.9% of T<sub>0</sub> positive plants. The *VIP1* gene was completely silenced in 13 out of 36 T<sub>1</sub> marker-free plants (36.1%). The *BAR* gene silencing in all positive plants happened in 27.3% of T<sub>1</sub> lines (6 out of 22 independent lines), with the *BAR* gene silenced in some positive plants in 9.1% of T<sub>1</sub> lines (2 out of 22 independent lines). This phenomenon needs to be confirmed in other studies in order to draw firm conclusions.

**Table 2** Genetic analysis of T<sub>1</sub> populations by molecular tests

| ID of T <sub>0</sub> plants tested | <i>VIP1</i> <sup>+</sup> <i>BAR</i> <sup>+</sup> | <i>VIP1</i> <sup>+</sup> <i>BAR</i> <sup>-</sup> | <i>VIP1</i> <sup>-</sup> <i>BAR</i> <sup>+</sup> | <i>VIP1</i> <sup>-</sup> <i>BAR</i> <sup>-</sup> |
|------------------------------------|--|--|--|--|
| SV17-1                             | 30   | 0  | 0  | 13   |
| SV19-6                             | 28   | 0  | 0  | 7  |
| SV17-10                            | 4  | 0  | 0  | 0  |
| SV17-2                             | 29   | 19   | 0  | 2  |
| SV20-4                             | 25   | 16   | 5  | 2  |
| SV21-1                             | 30   | 0  | 0  | 8  |
| SV20-2                             | 29   | 13   | 10   | 2  |
| SV18-1                             | 26   | 18   | 0  | 3  |
| SV21-2                             | 28   | 0  | 0  | 0  |
| SV18-3                             | 28   | 0  | 0  | 0  |
| SV19-9                             | 27   | 18   | 0  | 0  |
| SV17-5                             | 28   | 0  | 0  | 19   |
| SV17-9                             | 18   | 0  | 0  | 9  |
| SV18-2                             | 28   | 0  | 1  | 6  |
| SV22-1                             | 25   | 0  | 0  | 5  |
| SV20-8                             | 18   | 13   | 4  | 1  |
| SV17-11                            | 7  | 5  | 1  | 0  |
| SV25-1                             | 22   | 0  | 0  | 0  |
| SV28-2                             | 25   | 0  | 0  | 0  |
| SV17-14                            | 28   | 25   | 0  | 3  |
| SV18-10                            | 28   | 22   | 0  | 2  |
| SV19-17                            | 28   | 0  | 20   | 0  |
| total                              | 539  | 149  | 41   | 82   |

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