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Fertile Transgenic Asparagus Plants Produced by *Agrobacterium*-mediated Transformation

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Abstract

Transgenic plants of *Asparagus officinalis* cv. Mary Washington 500W were generated by *Agrobacterium*-mediated transformation. After induction of flowers by treatment with atrazine, calli were induced from male and female plants. Calli of male and female origin were co-cultivated with *Agrobacterium* EHA101 (pIG121Hm) and EHA101(pARK5), respectively. About thirty shoots developed from female calli and three shoots developed from male calli within six weeks on selective medium supplemented with 100 mg/*l* kanamycin and 500 mg/*l* claforan. Most of the shoots did not form roots. Seven shoots from female calli formed roots and were successfully transferred to soil in a greenhouse. But shoots of male calli did not form any roots. Stable integration and inheritance of transgenes were demonstrated by molecular and genetic analysis of transformants in the R_0 and R_1 generations. In an analysis using the polymerase chain reaction (PCR), fragments of both the *NPT* II gene and the *bar* gene were amplified from five R_0 plant fragments. Southern analysis showed that both *NPT* II and *bar* probes hybridized to products of PCR from five R_0 plants. One of the five R_0 plants was crossed with a non-transgenic male plant. The segregation ratio of transformed to non-transformed R_1 plants was about 1: 1. These results indicated that drug-resistant genes were transferred to *Asparagus* by *Agrobacterium*-mediated transformation.

1. Introduction

Asparagus (*Asparagus officinalis* L.) is a dioecious species in which sexual dimorphism is controlled by the genetic factors X and Y [1]. Male plants of this species are preferred for commercial production because of their greater yield, vigor and longevity. *Asparagus* is generally propagated from seed, and seeds are usually mixtures with 50% each of seeds of either sex [2]. It is impossible to distinguish the sex of plants before flowering. Abe *et al.* [3, 4] reported that one-month-old seedlings of *Asparagus* formed flowers when treated with atrazine (2-chloro-4-ethylamino-6-isopropylamino-1, 3, 5-triazine) or diuron [3-(3, 4-dichlorophenyl)-1, 1-dimethyl urea].

Monocotyledonous plants are considered generally to be insensitive to infection by *Agrobacterium*. However, some transgenic monocotyledonous plants have been reported. For example, *Asparagus* [5, 6, 7], rice [8, 9, 10, 11] and maize [12]. Transgenic *Asparagus* plants have also been produced by direct DNA uptake into protoplast [13] and particle gun bombarment [14]. However, an efficient *Asparagus* transformation system is still not available and information on progeny analysis is very sparse. Baochun and Wolyn reported the poor development and low germination rate of the hybrid seeds from crosses between the transgenic male *Asparagus* and non-transformed female *Asparagus* [14].

In this study, we attempted to transform male and female plants with drug-resistance genes by *Agrobacterium*-mediated transformation, and to investigate a segregation of the resistance in progeny.

2. Materials and Methods

2. 1 Plant material and preparation of calli

Seeds of *Asparagus officinalis* L. (cv. Mary Washington 500W) were surface-sterilized in 70% (v/v) ethanol for 30 seconds and then in 2% (v/v) sodium hypochlorite for 15 min. They were washed twice in sterile distilled water, placed on MS medium [15] supplemented with 100 mM atrazine for development of flowers, and cultured at 25°C for two weeks. After treatment with atrazine, the seeds were transferred to MS medium in culture bottles. Stem segments were cultured on MS medium supplemented with 0.5 mg/*l* 2, 4-dichlorophenoxyacetic acid (2, 4-D) to induce callus formation in the dark at 25°C. The calli obtained were sub-cultured on MS solid medium supplemented with 0.8% agar, 1 mg/*l* naphthaleneacetic acid (NAA) and 0.1 mg/*l* N^6-benzylaminopurine (BAP) every month.

2. 2 Bacterial strains, plasmids and transformation

Agrobacterium tumefaciens strain EHA101 [16] was used. Plasmids pIG121Hm and pARK5 were separately introduced into EHA101. pIG121Hm is a binary vector that contains a gene for neomycin phosphotransferase (*NPT* II) a gene for hygromycin phosphotransferase (*HPT*) and intron-gus in the T-DNA region. pARK5 contains a *NPT* II gene and bialaphos resistance gene (*bar*) in the T-DNA region.

Calli from male and female shoots were co-cultivated with EHA101(pIG121Hm) and EHA101 (pARK5) separately. Male and female calli, generated as described above, were cultured first on MS medium supplemented with 1 mg/*l* BAP and 0. 1 mg/*l* NAA for two days. *A. tumefaciens* was grown for one day in YEP medium. Next, half of the calli were immersed in the bacterial culture for 10 min and then transferred to MS medium supplemented with 1 mg/*l* BAP and 0. 1 mg/*l* NAA, and incubated at 25°C in darkness for 1, 2 and 3 days. After the co-cultivation, the materials were transferred to selection medium (MS medium plus 0. 8% agar, 1 mg/*l* BAP, 0. 1 mg/*l* NAA, 100 mg/*l* kanamycin and 500 mg/*l* claforan) at 25°C under continuous illumination (about 4 W/m²) and sub-cultured every 4 days for two weeks. After one month, regenerated shoots were transferred to rooting medium (MS medium plus 0. 8% agar and 100 mg/*l* kanamycin). Regenerated plants that had formed roots were eventually transferred to soil in pots and grown to maturity in a greenhouse.

2. 3 Analysis of DNA and the polymerase chain reaction (PCR)

Total DNA was prepared from transgenic *Asparagus* and non-transgenic *Asparagus* as described by Honda and Hirai [17]. To investigate of presence of the *NPT* II gene and the *bar* gene, an analysis was carried out by PCR with two pairs of primers (5′-CCCTCGGTATCCAATTAGAG-3′ and 5′-CGGGGGGTGGGCGAAGAACTCCAG-3′; 5′-GGATCCATGAGCCCAGAA-3′ and 5′-TCAGATCTCGGTGACGGGCA-3′). PCR was performed for 35 cycles, with each cycle consisting of 45 sec. at 94°C, 30 sec. at 55°C and 90 sec. at 72°C. The reaction mixture was finally incubated for 10 min. at 72°C. Products of PCR were separated by electrophoresis on an 0. 8% (w/v) agarose gel and bands of ethidium bromide-stained fragments were visualized with a UV illuminator.

Southern hybridization was carried out with a Non-radioactive DNA Labeling and Detection Kit (Boehringer Mannheim, Germany). Fragments of the *NPT* II gene and the *bar* gene were used as probes for Southern hybridization.

3. Results

3. 1 Selection of male and female plants

Seeds of *Asparagus officinalis* cv. Mary Washington 500W were treated with 100 mM atrazine in aseptic culture for two weeks, and then transferred to MS solid medium. After one month, eight plants (15%) out of a total of 52 had flowered; six shoots were female with degenerated stamens and two shoots were male with degenerated pistils (**Table. 1**). Fragments of the stems of these plants were cultured on MS solid medium supplemented with 0. 5 mg/*l* 2, 4-D to induce formation of callus. Calli that formed were transferred to MS solid medium supplemented with 1 mg/*l* NAA and 0. 1 mg/*l* BA. These calli were used for transformation.

3. 2 Transformation

After two days of pre-culture, female calli of about 2-3 mm in diameter were co-cultivated for 1, 2 or 3 days with *Agrobacuterium tumefaciens* EHA101 that harbored plasmid pARK5, which contained a *bar* gene and a gene for neomycin phosphotransferase (*NPT* II). Male calli were similarly co-cultivated with *A. tumefaciens* EHA101 that harbored plasmid pIG121Hm, which contained intron-gus, *NPT* II and a gene for hygromycin phosphotransferase (*HPT*). Then the calli were cultured on MS solid medium supplemented with 1 mg/*l* BAP, 0. 1 mg/*l* NAA, 100 mg/*l* kanamycin and 500 mg/*l* claforan. After one month, five male calli and 11 female calli were shown to be resistant to kanamycin. Most of them had been obtained from 2-day co-cultured calli (**Table 2**). Although many shoots were regenerated from selected female calli (**Fig. 1-A**), most of them did not

Table 1. Rates of flowering of male and female plants induced by 100 mM atrazine treatment.

Plants	Flowering plants	Males	Females
52	8 (15%)*	2 (4%)	6 (11%)

* Percentages are shown in parentheses.

Table 2. Numbers of selected cells on meduim supplemented with 100 mg/*l* kanamycin.

Duration of co-culture (days)	Female	Male
1	2(111)*	1(102)
2	8(98)	4(89)
3	1(85)	0(102)

* Numbers in parentheses are total numbers of celli incubated with *Agrobacterium*.

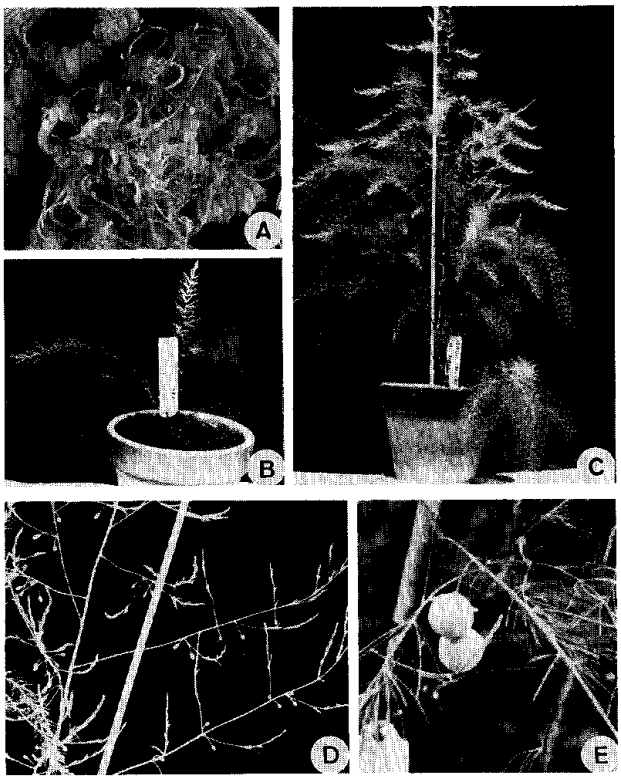

Fig. 1 A, Female shoots regenerated from selected calli.
B, A female transgenic plant in soil.
C, A female transgenic plant after three months of growth in the greenhouse.
D, Flowering of a transgenic female plant.
E, Seeds of transgenic female plant obtained by crossing with non-transgenic male plant.

form roots. By contrast, eight shoots were regenerated from selected male calli, but none formed any roots. Finally, seven plants (R_0) obtained from female calli were successfully moved to the greenhouse (**Fig 1-B, C**).

3. 3 Analysis of Ro plants

Total DNA was extracted from leaves of the seven R_0 plants and of non-transformed plants. Amplification was performed by PCR with two pairs of primers, as described in Materials and Methods. When the primers for amplification of the *NPT* II gene were used, five out of seven plants yielded amplified fragments of about 1.0 kbp, as did plasmid pARK5 when it was used as a positive control template. The identity of the amplified *NPT* II gene was confirmed by Southern hybridization (**Fig. 2**). When primers for amplification of the *bar* gene were used, five of seven plants again yielded amplified fragments of about 700 bp, as did plasmid pARK5 when it was used as a positive control template. The identity of the amplified *bar* gene was also confirmed by Southern hybridization (**Fig. 3**).

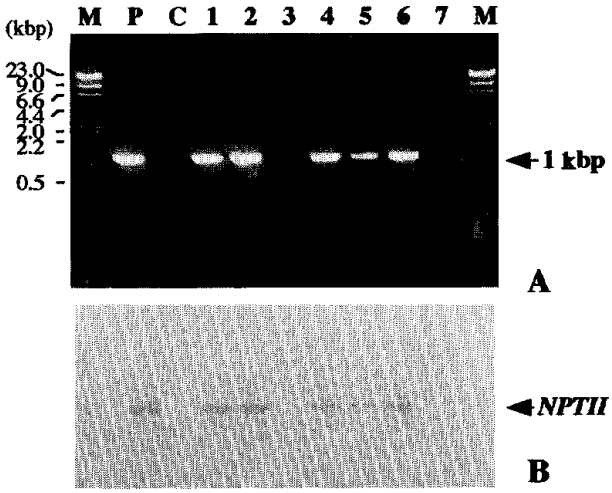

Fig. 2 Analysis by PCR of genomic DNAs and Southern blotting analysis products of PCR from transgenic (R_0) plants.
A, Genomic DNA was amplified by PCR from plasmid pARK5 (control; lane P), from a non-transformed control (lane C) and from seven transgenic plants (lanes 1-7). Lane M indicates λ size marker digested with *Hind*III. The ethidium bromide-stained gel shows PCR-amplified 1.0-kbp fragments of *NPT* II.
B, Southern blotting analysis of the products of PCR using the *NPT* II gene as probe.

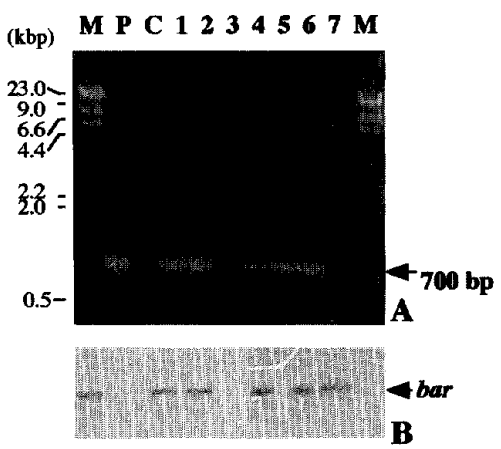

Fig. 3 Analysis by PCR of genomic DNAs and Southern hybridization analysis of products of PCR from transgenic (R_0) plants.
A, Genomic DNA was amplified by PCR from a plasmid pARK5 (control; lane P), a non-transformed control (lane C) and seven transgenic plants (lanes 1-7). Lane M indicates λ size-marker digested with *Hind* III. The ethidium bromide-stained gel shows PCR-amplified 700-bp fragments of the *bar* gene.
B, Southern blotting analysis of products of PCR using the *bar* gene as probe.

3. 4 Analysis of R_1 plants

One of the five plants discussed above flowered (**Fig. 1-D**) and it was crossed with a non-transgenic

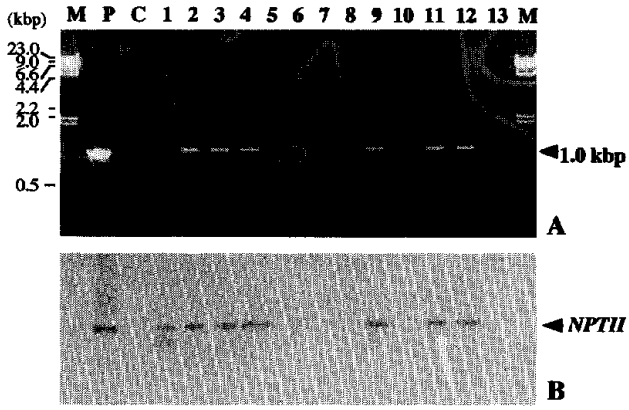

Fig. 4 Analysis by PCR of genomic DNAs and Southern hybridization analysis of products of PCR from transgenic (R_1) plants.

A, Genomic DNA was amplified by PCR from a plasmid pARK5 (control; lane P), a non-transformed control (lane C) and thirteen transgenic plants (lanes 1-13). Lane M indicates λ size marker digested with *Hind* III. The ethdium bromide-stained gel shows PCR-amplified 1.0-kbp *NPT* II fragments.

B, Southern blotting analysis of products of PCR using the *NPT* II gene as probe.

male plant. The resultant seeds (R_1, **Fig. 1-E**) were germinated at 25°C in darkness and seedlings were grown in soil in the greenhouse. In an analysis of 13 R1 plants by PCR, seven plants yielded amplified fragments, whose identities were confirmed by Southern hybridization (**Fig. 4**). The segregation ratio for transformed and non-transformed R_1 plants was about 1: 1. These results indicated a single locus inherited in a Mendelian fashion.

4. Discussion

In this study, we tried to transform male and female *Asparagus* with drug-resistance genes. We obtained transgenic female plants but no transgenic male plants. A possible explanation was that the regenerative ability of females was higher than that of males under the conditions used in this study. We wanted to select male seedlings transformed *bar* gene on medium supplemented with bialaphos and female seedlings transformed *HPT* gene on medium supplemented with hygromycin to allow early and easy discrimination of the sex of *Asparagus*. This is the first attempt of the *Agrobacuterium tumefaciens*-mediated transformation with male and female calli of *Asparagus*, respectively and of the analysis of progeny (R_1). In transgenic *Asparagus* plants obtained through particle gun bombardment [14], hybrid seeds of crosses between the transgenic male *Asparagus* and non-transgenic female *Asparagus* were poor development and low germination rate. It is thought that the poor seed development could have arisen from chromosomal abnormalities occurring with the plant tissue culture process for the transgenic plants or from the incorporation of foreign DNA into the *Asparagus* genome. However, most of R_1 progeny obtained in this study showed high germination rate and good development. Since one problem in this study was the low regeneration rate of male calli, further work is needed to improve the efficiency of this transformation system. Male and female transgenic plants should be useful materials not only for early discrimination of sex, but also for analysis of sex-determination factors and they should provide selective markers for cell fusions.

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