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Recovery of herbicide-resistant Azuki bean [*Vigna angularis* (Wild.), Ohwi & Ohashi] plants via *Agrobacterium*- mediated transformation

Mutasim M. Khalafalla[1,2]*, Hany A. El-Shemy[1,3], Rahman S. Mizanur[1], Masayoshi Teraishi[1], Masayoshi Teraishi[1], Masao Ishimoto[1]

[1]National Agricultural Research Center for Western Region, 6-12-1 Nishifukatsu, Fukuyama, Hiroshima 721-8514, Japan. [2]Current address: Commission of Biotechnology and Genetic Engineering, National Center for Research, Khartoum, Sudan.

[3]Current address: Faculty of Agriculture, Cairo University, 12613 Giza, Egypt.

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Transgenic azuki bean [*Vigna angularis* (Willd.) Ohwi & Ohashi] plants expressing the hygromycin phosphotransferase (*hpt*), green fluorescent protein (*sgfp*) and phosphinothricin acetyltransferase (*bar*) genes were obtained by *Agrobacterium- tumefacients* - mediated transformation. A total of 210 epicotyl explants were inoculated with *A. tumefaciens* strain EHA105, harboring the binary plasmid pZHBG on MS co-cultivation medium supplemented with 100 mM acetosyringone and 10 mg/l of BA. Following selection on MS medium with 15 mg/l of hygromycin, the regenerated adventitious shoots that formed on the induced calli were further screened for *sgfp* expression before transferred to rooting medium. 31 transgenic plants were obtained with transformation frequency of 14%. The presence of transgenes in transformed azuki bean plants was confirmed by polymerase chain reaction (PCR) and southern blot analysis. Transcription of the *bar* and *hpt* genes was assessed by reverse transcription polymerase chain reaction (RT-PCR) analysis. *sgfp*- positive transgenic plants exhibited functional expression of the *bar* gene as determined by assaying for resistance to bialaphos applied directly to leaves. This result demonstrates the feasibility of introducing potentially useful agronomic traits into azuki bean through genetic engineering.

Key words: *Agrobacterium tumefaciens*, *bar* gene, bialaphos, transgenic, *Vigna angulazris*.

INTRODUCTION

Azuki bean [*Vigna angularis* (Willd.) Ohwi & Ohashi] is an edible grain legume grown and used for centuries primarily in the East Asian countries of Japan, Korea, China, and Taiwan. Azuki bean seed is principally used in confectionery products. Seeds are cooked and mixed with varying portions of sugar and other ingredients to produce *an*, which is used as pastry filling in traditional Oriental confections (Breene and Hardman, 1989; Hang et al., 1993; Lumpkin et al., 1993). Botanically, azuki bean belong to the tribe *Phaseoleae* which, includes other staple grain legume crops such as soybean, common bean, cowpea and mungbean, all of which are major protein or oil sources. Among the tribe *Phaseoleae*, azuki bean is not one of the cosmopolitan crops but it has a reproducible and efficient *Agrobacterium*-mediated transformation system (Yamada et al., 2001; El-Shemy et al., 2002). The majority of legume transformation studies have favored the use of *Agrobacterium tumefaciens* to generate transgenic soybeans (Hinchee et al., 1988; Chee et al., 1989), chickpeas (Fontana et al., 1993) and pea (Puonti-Kaerlas et al., 1990, 1992; De Kathen and Jacobsen 1990; Davies et al., 1993; Schroeder et al., 1993; Shade et al., 1994; Zubko et al., 1990).

*Correspondence author. E-mail: mutasim@myway.com.

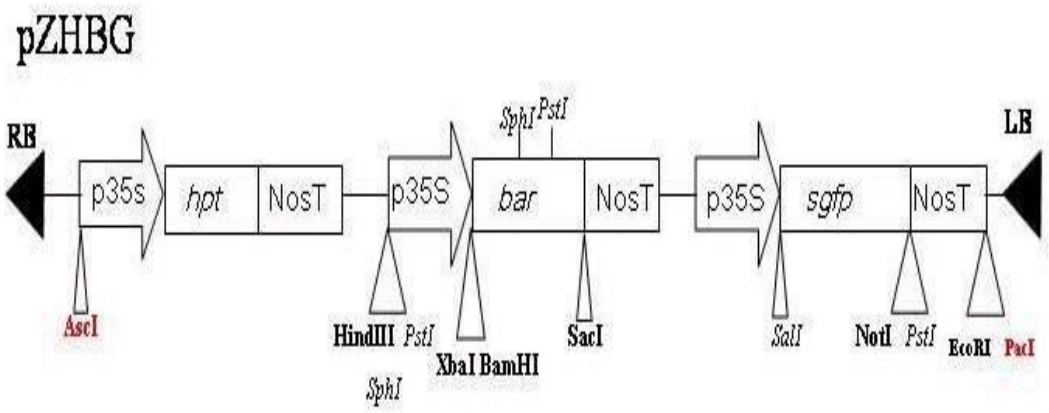

Figure 1. Schematic representation of binary plasmid pZHBG. RB: Right border; CaMV35S: cauliflower mosaic virus 35S promoter; *hpt*: hygromycin phosphotransferase gene; Tnos: nopaline synthase gene terminator; *bar*: a bialaphos resistance gene from *S. hygroscopicus*; *sgfp*: green florescent protein gene.

Efficient transformation system provide the initial impetus for the genetic modification of azuki bean and therefore, its value and productivity it might be further improved by introducing foreign genes responsible for improving protein quality, pest and disease resistance and herbicide resistance. Moreover, azuki bean could be emerged as an important leguminous model plant to provide the framework within which the molecular mechanisms that underlie the grain legume-specific character can be clarified and leguminous crops in general can be improved.

In recent years, considerable progress has been made in genetic engineering of various plant species, both agronomically important crops as well as model plants. The bases of this progress were, in addition to efficient transformation methods, are the designs of appropriate signals regulating transgene expression and the use of selection marker and reporter genes. In most cases, a gene of interest is introduced into plants in association with a selectable marker gene. Recovery of a transgenic plant is, therefore, facilitated by selection of putative transformants on a medium containing a selection agent, such as antibiotic, antimetabolite and herbicide. On the other hand, use of reporter genes (CAT, lacZ, uidA, luc, gfp) allows not only distinguishing transformed and non-transformed plants, but first of all to study regulation of different cellular processes. In particular, by employing vital markers (Luc, GFP) gene expression, protein localization and intracellular protein traffic can be now observed *in situ*, without the need of destroying plant.

In this study we constructed a binary plasmid (pZHBG) comprising the *bar* gene encoding the enzyme, phosphinothricin acetyltransferase which directly inactivates the herbicides phosphinothricin and confers resistance to the commercial herbicides, bialaphos (Confaloneiri et al., 2000), which was fused between the reporter gene s*gfp* (green fluorescent protein) and selectable marker *hpt* gene (hygromycin phosphotransferase).We employed this plasmid to develop an efficient *Agrobacterium*- mediated transformation protocol that would favor the minimal survival of non-transformants and production of transgenic herbicide resistant azuki bean plant.

MATERIALS AND METHODS

Plant material

Seeds of azuki bean cultivar Beni-dainagon were obtained from Hokkaido Tokachi Agricultural Experimental Station, Memuro, Hokkaido, Japan.

Agrobacterium strain and plasmid

A. tumefaciens strain EHA105 containing the disarmed hypervirulent plasmid pTiBo542 in the C58 chromosomal background (Hood et al., 1993) was used for transformation. A dual marker binary plasmid pZHG comprising the reporter gene s*gfp* (green fluorescent protein) and *hpt* gene (hygromycin phosphotransferase) as a selective marker with a multiple cloning site between them was constructed based on pZH2 vector which is a derivative of pPZP202 (Hajdukiewicz et al., 1994). In pBI221 (Clontech, Franklin Lakes, NJ USA) the *bar* gene encoding resistance to bialaphos fused between the CaMV35S promoter and NOS terminator was excised along with the CaMV35S promoter and NOS terminator with EcoRI, filled in with T4 DNA polymerase (Takara Bio, Otsu, Japan) and then digested with *HindIII* and purified. The fragment (2.1kb) was ligated into the multiple cloning site of the pZHG to create the new binary plasmid, hereafter referred to as pZHBG (Figure 1). The pZHBG (13.4Kb) was subsequently transferred to *A. tumefaciens* by electroporating at 2500 V.

Plant transformation, selection and regeneration *Agrobacterium*

mediated transformation system of azuki bean

(*vigna angularis* Ohwi & Ohashi), was optimized according to the methods previously described by Yamada et al. (2001). Dry seeds of a cultivar, Beni-dainagon, were sterilized in 70% ethanol for 30 s followed by 1% sodium hypochlorite for 15 min and two washes with sterile distilled water. Seeds were germinated at 25°C in the dark condition on MS basal medium (Murashige and Skoog, 1962), containing 30 g/l sucrose and 8 g/l agar (Wako Pure Chemical Industries) (pH 5.8). Ten days after plating, elongated epicotyls of etiolated seedlings were cut into pieces about 10 mm long with a scalpel blade, and 20 to 30 explants were plated sideways on co-cultivation medium in 90 x 20 mm Petri plate. Co-cultivation was conducted on the MS media containing 30 g/l sucrose, 8 g/l agar, 10 mg/l BA and 100 µM acetosyringon*. A. tumefaciens* strain EHA105 from a plate colony was grown at 28°C overnight in liquid LB broth containing 100 µg/ml kanamycin. Bacterial cells were collected by centrifugation and resuspended to a final OD_{600} of 0.1 to 0.2 in MS liquid medium containing 15 g/l glucose, and 2 µl of the suspension was placed on the wounded site of each explant. After 2 days of co-cultivation at 25°C in the dark, explants were washed twice with MS liquid medium. Excess liquid of explants was withdrawn with sterile filter paper. Explants were plated on MS medium containing 1 mg/l BA, 15 mg/l hygromycin, and 500 mg/l lilacillin (Takeda Chemical Industries), and incubated at 25°C under cool white fluorescent light (16/8 light regime, 50- 60 $\mu molm^{-1}s^{-2}$). The explants were transferred to fresh medium at two weeks intervals. When adventitious shoots developed and after more than 2 leaves emerged from shoots, each shoot was excised and transferred to MS medium without plant growth regulators but containing 15 mg/l hygromycin and 500 mg/l lilacillin. Rooted shoots were excised and repeatedly selected on the same medium.

GFP detection

The presence of *sgfp* (S65T) was detected by blue light excitation (Chiu et al., 1996). Adventitious shoots developed from the explants and leaves emerged from shoots were observed with a fluorescent microscope with a filter set providing 455-490 nm excitation and emission above 515 nm.

PCR and southern blot analysis

Total DNA was isolated from both untransformed control plantlets and GFP-positive plantlets of azuki bean by the method of Draper and Scott (1988), and used to investigate the presence of the introduced gene(s). PCR analysis was conducted to screen putatively transformed plants in a 20 µl reaction mixture containing 10 ng of genomic DNA, 200 µM of each of dNTP, 0.2 µl of each primer, and 2.5 U of Ampli- taq Gold polymerase (PE Biosystems) in the corresponding buffer. Reactions were hot -started (9 min at 94°C) and subjected to 30 cycles as fellows: 30 s at 94°C; 1 min at 58°C; and 1 min at 72°C. The last extension phase was prolonged 7 min at 72°C. The primer sets for *hpt* were designed for amplification of the 560 bp fragment; sequences are:

5'-ATCCTTCGCAAGACCCTTCCT-3' and
5'-GGTGTCGTCCATCACAGTTTG-3'.

The primers for *sgfp* were designed for amplification of the 708 bp fragment; sequences are:

5'-AGCAAGGGCGAGGAGCTGGTT-3' and
5'-CTTGTACAGCTCGTCCATGC-3'.

The primers for *bar* gene were designed for amplification of the 510 bp fragment; sequences are

5'-CGGGGATCCTCTGGATCTTC-3' and
5'-GACTCTAGAGGGGCATGACC -3'.

Southern blot analysis was conducted to confirm stable integration of transgenes, 6 µg of total DNA was digested with the restriction enzymes, *HindIII* and digested DNA was separated by electrophoresis in a 1% agarose gel and transferred onto hybond N+ membrane (Amersham Pharmacia Biotech). Labeling and detection were conducted following the protocol of ECL direct nucleic acid labeling and detection (Amersham Pharmacia Biotech). DNA fragment containing the *bar* gene was amplified from the plasmids by the same primer sets for the PCR analysis, and used as hybridization probe on Southern blot membrane.

RNA isolation and RT-PCR

Total RNA from leaves of azuki bean was isolated from both transgenic and untransformed control plants. The leaves (100 mg) were immersed in liquid nitrogen, ground to a fine powder with a mortar and pestle, after which 1 ml of Trizol reagent (GIBCO-BRL) was added and the ground samples were shaken vigorously. The samples were incubated at room temperature for 5 min and, after the addition of 200 µl of chloroform, centrifuged at 15,000 g for 10 min. Total RNA was precipitated from the resulting supernatant with isopropanol and resuspended in 100 µl of RNase-free water. A portion of the RNA (500 ng) was incubated for 30 min at 37°C with 3 U of RNase-free DNase (RQ1; Promega, Madison, WI, USA) in 30 µl of a solution containing 40 mM Tris-HCl (pH 8.0), 10 mM MgSO4, and 1 mM CaCl2. The reaction was stopped by the addition of 3 ul of 20 mMEGTA (pH 8.0), after which the RNA was subjected to phenol -chloroform extraction and ethanol precipitation. The isolated RNA was resuspended in RNase-free water and subjected to first-strand cDNA synthesis with a ThermoScript RT-PCR System (Invitrogen) and an oligo dT 20 primer. The RNA was thus incubated first for 5 min at 65° C in 12 ul of a solution containing 50 nmol of the primer and 20 nmol of each dNTP and then, after the addition of 8 ul of cDNA synthesis mix comprising 4 ul of 5 cDNA synthesis buffer, 0.1 nmol of dithiothreitol 40 U of RNase-out, and 15 U of Thermo Script reverse transcriptase for 60 min at 50°C The resulting cDNA was subjected to PCR with AmpliTaq Gold DNA polymerase (Applied Biosys-tems) for 30 cycles of 94° C for 1 min, 55° C to 60°C for 1 min, and 72° C for 1 min. Amplification products were cloned into the pCR2.1 vector (Invitrogen) and sequenced for product confirmation. The PCR primers (forward and reverse, respectively) were as follows:

5'-CGGGGATCCTCTGGATCTTC- 3' and
5'-GACTCTAGAGGGGCATGACC -3'.

The *Actin1* gene served as an internal control for RT-PCR

Transfer of plantlets to soil and herbicide resistance

After substantial root growth, the plants were directly transplanted to soil in the greenhouse. Two month later fully expanded leaves of each transgenic and untransformed control plants were wetted with 500-1000 mg/l bialaphos solution including 0.1% Triton 100-X as surfactant. After 1-2 weeks, the effect of the herbicide on the treated leaves was investigated.

RESULTS AND DISCUSSION

Transformation, selection and regeneration of transgenic plants

A total of 210 epicotyl explants were inoculated with *A.*

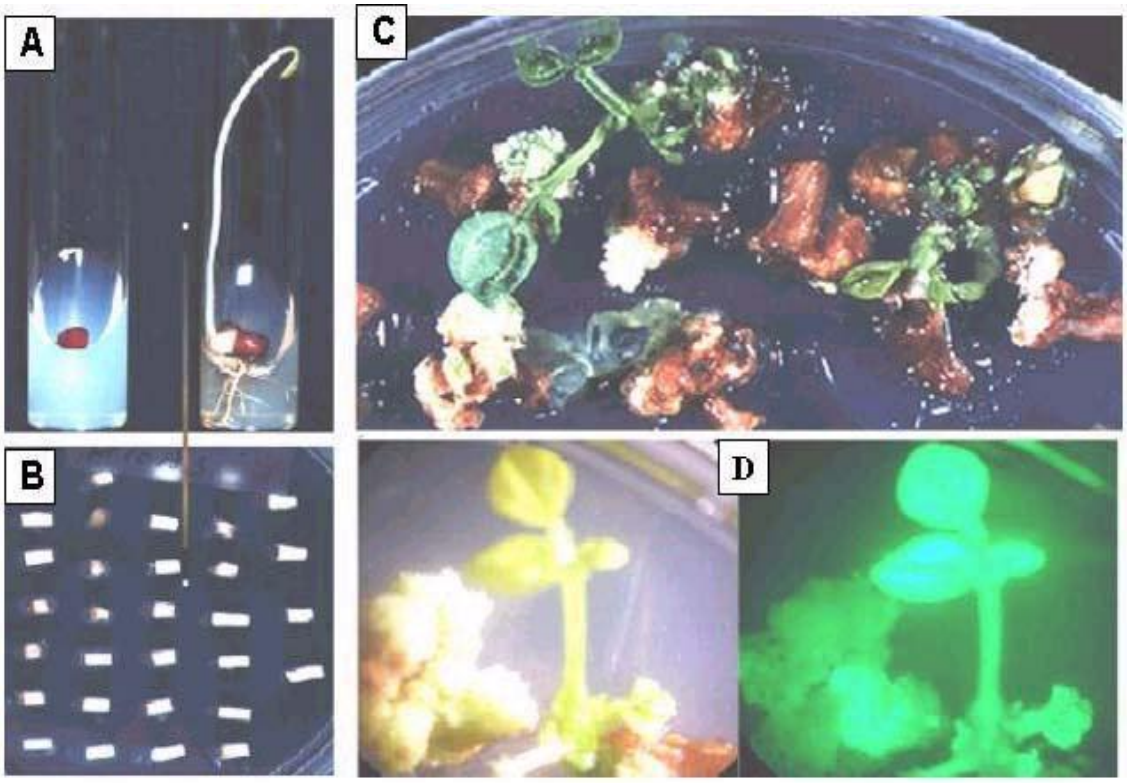

Figure 2A- D. Genetic transformation of azuki bean. (A) Seed germination (left) and elongated epicotyle in dark (right). (B) Co-cultivation of epicotyl explants with *Agrobacterium* strain EHA105. (C) Shoot regeneration from organic calli induced on the selection medium. (D) Transgenic shoot screened for GFP expression under white light (left) and blue Light (right).

Table 1. Azuki bean *Agrobacterium*-mediated transformation frequency during different steps of analysis.

Procedure	Number of explants, shoots or plantlets	Frequency (%)
Explants inoculated	210	
Shoots regenerated in medium containing hygromycin	94	44.76
Shoots showed *sgfp* expression under blue light excitation	46	21.90
Shoots rooted in medium containing hygromycin	31	14.76
hpt - positive (PCR)	31	14.76
sgfp - positive (PCR)	31	14.76
bar gene – positive (PCR)	31	14.76
Putative stable transformation events	31	14.76

tumefaciens strain EHA105, harboring pZHBG on the co-cultivation medium (MS medium supplementedwith 100mM acetosyringone and 10 mg/l of BA) (Figure 2B). After 2 days of co-cultivation, the explants were placed on a regeneration medium (MS medium with 1 mg/l of BA) supplemented with 15 mg/l of hygromycin. The explants were transferred to a fresh regeneration medium every two weeks. Almost all the explants formed white calli on the selective medium. All the adventitious shoots that formed on the induced calli (Figure 2C) were further screened for *sgfp* expression (Figure 2D) before transferred to rooting medium (MS medium without plant growth regulators supplemented with 15 mg/l of hygromycin). Genetic transformation of azuki bean has been greatly facilitated by the combined action of acetosyringone and high concentration of BA in co-cultivation medium (Yamada et al., 2001). From 94 adventitious shoots only 46 shoots (48.9%) (Table 1) that

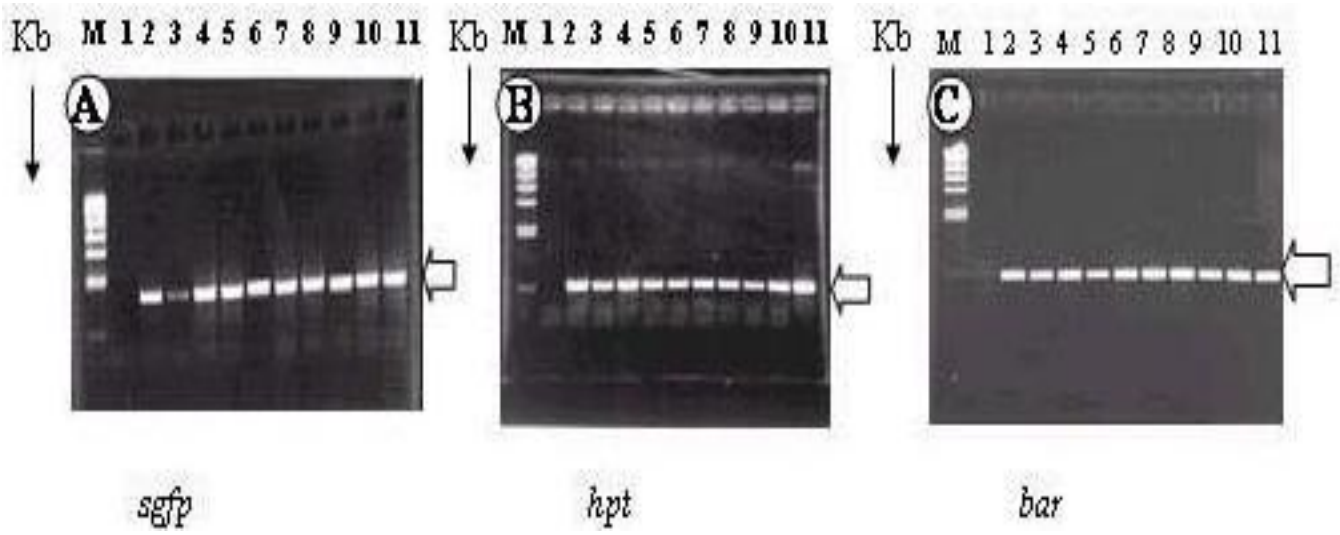

Figure 3A-C. Detection of foreign genes in transgenic azuki bean plants by PCR: *sgfp* (A), *hpt* (B) and *bar* (C) genes. M: molecular standards of λ DNA digested with *HindIII*; Lane 1: non- transformed azuki bean plant; Lanes 2-11: transformed azuki bean plants. Arrowheads expected 708 bp, 560 bp and 510 kb fragments of *sgfp*, *hpt* and *bar* genes, respectively.

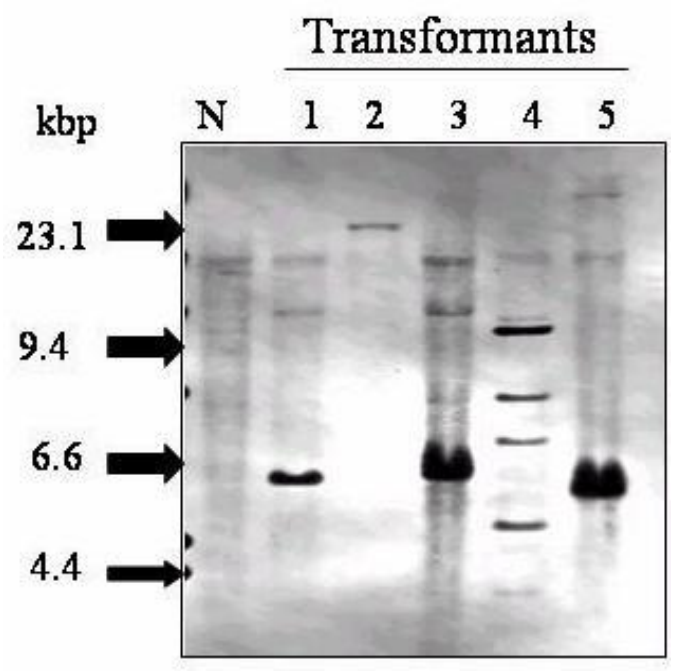

Figure 4. Southern blot analysis of genomic DNA isolated from azuki bean transformants. A membrane was hybridized with *bar* specific brobe. N: Non-transformed (control). Lanes 1 to 5: transfgormants. Arrows at left indicate DNA standard fragment size.

showed *sgfp* expression were excised and transferred to a rooting medium. Explants were allowed to form adventitious shoots for 3 months after the co-cultivation. 31*sgfp*-expressed shoots were eventually obtained with root formation on selective medium (Table 1). The screening of the regenerated shoots for *sgfp* expression before transferring to rooting medium is highly beneficial in minimizing the survival of non-transformants and guarantee only transgenic shoots to be rooted and hence save the cost and time at this stage. All the regenerated plants (T0) grew, flowered, and produced seeds normally.

Molecular analysis of putatively transformed azuki bean

Putatively transgenic azuki bean plants were first tested by polymerase chain reaction (PCR) analysis to verify the presence of *the bar, hpt*, and *sgfp* genes. The PCR analysis clearly confirmed the presence of the three foreign genes in all 31 *sgfp*- expressed shoots rooted in medium containing hygromycin (Table 1). The PCR products amplified from transgenic plants DNA had the expected size of 510, 560 and 708 bp for *bar*, *hpt* and *sgfp* genes, respectively. No amplified bands were observed in PCR products from total DNA of untransformed control plants. Southern blot analysis was carried out to evaluate further the insertion of the *bar* gene in the genomes of the *sgfp*-positive plantlets. The total DNA of some PCR -positive azuki bean plants was digested with *HindIII* and hybridized with the probe for *bar* gene. The results showed that the transgenic plants yielded 1 to 4 bands hybridized with *bar* probe, but non-transgenic plants did not yield any band. All of these plantlets showed a hybridization bands with the *bar* probe suggested the insertion of a 1- 4 copies of the gene (Figure 4). Multiple site integrations of transgenes were

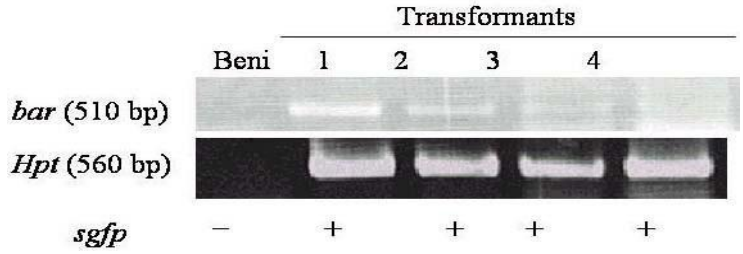

Figure 5. RT-PCR analysis of the *bar* and *hpt* genes in control untransformed azuki bean Plant (lane 1) and transformants (lanes 2-5). Presence (+) and absence (-) of *sgfp* gene in the same plants also indicated.

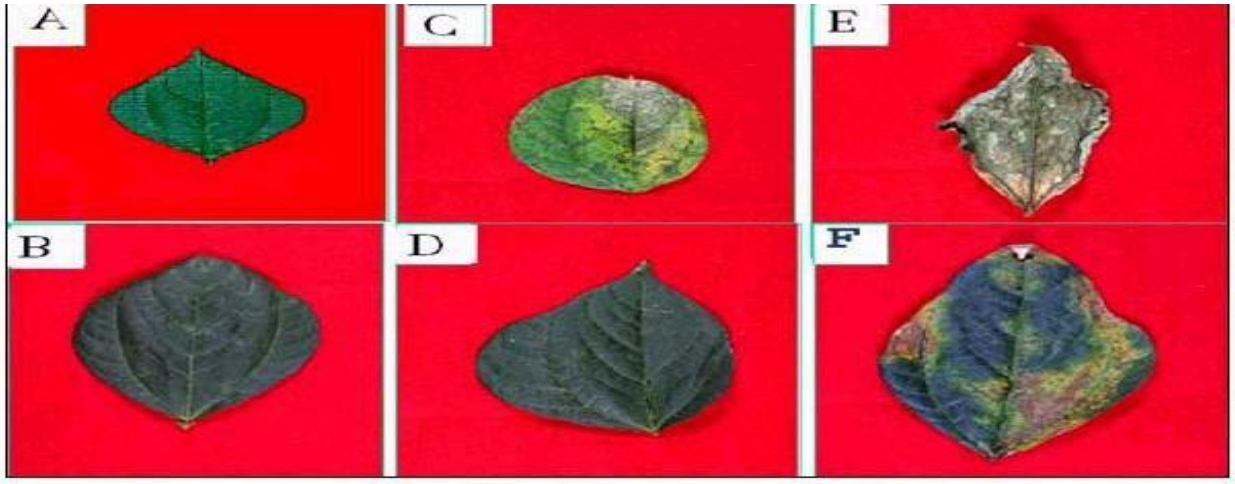

Figure 6A-F. Evaluation of herbicide resistance in control and transgenic azuki bean leaves under green house conditions. A: control untransformed leaf without bialaphos. B: transgenic leaf without bialaphos. C: untransformed leaf treated with bialaphos at 1 mg/l. D: transgenic leaf treated with bialaphos at 1 mg/l. E: untransformed leaf treated with bialaphos at 2 mg/l. F: transgenic leaf treated with bialaphos at 2 mg/l.

already reported in azuki bean (Yamada et al., 2001; El-Shemy et al., 2002)

The expression of the transferred genes was studied by reverse transcription polymerase chain reaction (RT-PCR). RT-PCR analysis using *hpt* and *bar* genes showed an amplification of 560 bp for *hpt* and 510 for the *bar* gene in the four transgenic plants, while the control plant showed no amplification. This result clearly confirmed that both *hpt* and *bar* genes are transcriptionaly active in azuki bean genome. All bialaphos-resistant transgenic plants showed detectable levels of *bar* mRNA transcripts.

In vivo herbicide application assay

A preliminary experiment was carried out to determine the dose response of azuki bean plant to bialaphos. Our objective was to ascertain bialaphos levels that would allow for differential growth between transformed and non-transformed plants. Bialaphos levels ranging from 0.025 up to 2.0 mg/ml were tested. A total of 6 plants were evaluated per bialaphos level per experiment. Non-transformed azuki bean plants were very sensitive to the bialaphos and all plants sprayed with bialaphos level greater than 0.025 mg/ml they showed signs of necrosis and chlorosis on basal leaves under normal growth conditions and died 2-10 days (depending on bialaphos concentration) after the beginning of the treatment (data not shown). We decided to use 2 levels of 1.0 and 2.0 mg/ml bialaphose for evaluation of herbicide resistance in transgenic azuki bean plants.

The response of transformants to herbicide (bialaphos) applied directly to leaves provides convincing evidence for functional activity of the transgene (Knapp et al., 2000) . The application of bialaphos at 1.0 mg/ml directly to the leaves of the control plant of azuki bean resulted in necrosis and turned brown and eventually died after one week under normal growth conditions (Figure 6C, D). In

contrast, treated leaves of transgenic plants remain green and healthy (Figure 6 D). At higher concentration of bialaphos (2 mg/ml) treated leaves of the control become necrosis and turned brown after 3 days (Figure 6E). However, in case of transgenic plants the tissue of the leaves to which bialaphos applied partially become necrotic but the symptoms did not spread and the plants eventually recovered fully (Figure 6F) . All transgenic azuki bean obtained in this experiment appeared normal and were morphologically indistinguishable from control plants.

In this study we have demonstrated for the first time the production of azukin bean transgenic plant with resistance to herbicide. This resistance could permit control of weed competition during the establishment of azuki bean in the field and lead to reduction of deleterious environmental effects due to the application of high doses of toxic herbicides.

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