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Agrobacterium-mediated transformation of faba bean *(Vicia faba* L.*)* using embryo axes

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Summary

A system for the production of transgenic faba bean by *Agrobacterium*-mediated transformation was developed. This system is based upon direct shoot organogenesis after transformation of meristematic cells derived from embryo axes. Explants were co-cultivated with *A. tumefaciens* strain EHA105/pGlsfa, which harbored a binary vector containing a gene encoding a sulphur rich sunflower albumin (*SFA8*) linked to the *bar* gene. Strain EHA 101/pAN109 carrying the binary plasmid containing the coding sequence of a mutant aspartate kinase gene (*lysC*) from *E. coli* in combination with neomycinphosphotransferase II gene (*nptII)* was used as well. The coding sequences of *SFA8* and *LysC* genes were fused to seed specific promoters, either *Vicia faba* legumin B4 promoter (LeB4) or phaseolin promoter, respectively. Seven phosphinothricin (PPT) resistant clones from Mythos and Albatross cultivars were recovered. Integration, inheritance and expression of the transgenes were confirmed by Southern blot, PCR, enzyme activity assay and Western blot.

Abbreviations: 2,4-D: 2,4-dichlorophenoxyacetic acid; BAP: 5-benzylaminopurine; LB: T-DNA left border; *lysC*: aspartate kinase gene; MS: Murashige & Skoog; NAA: naphthylacetic acid; *nptII*: neomycinphosphotransferase II gene; RB: T-DNA right border; *SFA8*: sunflower albumin8 gene

Introduction

Faba bean (*Vicia faba* L.) is the main grain legume grown for dry seeds for human consumption and animal feed in many of the developed and developing countries especially in the West Asian and North Africa region (WANA). Difficulties in pollination control and a narrow genetic base imposed limitations for the varietal improvement of this species by conventional breeding methods (Bond, 1987). It is in this context that genetic transformation may be one of the methods of choice for the improvement of this important crop. *V. faba* exhibits, like most other grain legumes, a rather low competence for *in vitro* culture, mainly due to difficulties in the regeneration from callus tissues and the high content of phenolic compounds which cause cell death (Selva et al., 1989; Bieri et al., 1984).

To date, there is only a single report on *Vicia faba* cv. 'Mythos' transformation using *Agrobacterium* mediation where fertile transgenic plants were recovered (Böttinger et al., 2001). This *Agrobacterium*-based transformation protocol makes use of the *de novo* regeneration of shoot initials from callus that was initially developed for plant regeneration from protoplasts (Tegeder et al., 1995). Transgenic plants were recovered by inoculation of stem segments with *Agrobacterium* EHA 101 or EHA 105, harboring different binary vectors, followed by callus induction

on MS (Murashige & Skoog, 1962) medium containing 0.5 mg/l of each thidiazuron (TDZ), 2,4-dichlorophenoxyacetic (2,4-D) and naphthylacetic acid (NAA) and 100 mg/l kanamycin as a selective agent. Afterwards transgenic shoots were regenerated *via* organogenesis using a high concentration of thidiazuron (7.5 mg/l) and 0.75 mg/l NAA. Finally the plants were recovered by micro-grafting. This process is however time consuming, requires 16–24 months to get seed producing primary transformants, has a relatively low efficiency and is prone to somaclonal variation.

In an effort to improve *Vicia faba* transformation efficiency, we have investigated different transformation systems using either mature or immature zygotic embryo axes as a target for *Agrobacterium*-mediated transformation. We hereby report, for the first time, the possibility of using *A. tumefaciens* mediated transformation with zygotic embryo axes to regenerate stable fertile transgenic plants using two faba bean cultivars, Mythos and Albatross.

Materials and methods

Plant material

Six selected cultivars of *Vicia faba* i.e. Mythos, Albatross (obtained from Norddeutsche Pflanzenzucht/W.Lemke, Hohenlieth, Germany), Giza 2, Giza 429, Giza blanka and Giza 716 (obtained from Agricultural Research Center ARE, Egypt) were used.

Agrobacterium strains and plasmids

Agrobacterium-mediated transformation experiments were conducted using *A. tumefaciens* strain EHA 105 harboring binary plasmid pGlsfa (pGPTV-bar derivative, Becker et al., 1992) containing *SFA8* gene under the seed specific leguminB promoter (LeB4) and the *bar* gene. The selectable marker *bar* gene of *Streptomyces hygroscopicus* encodes phosphinothricin acetyltransferase (PAT), which inactivates phosphinothricin (PPT), the active component of BASTA by acetylation (Thompson et al., 1987). *Agrobacterium* strain EHA 101 carrying the binary vector pAN109 (kindly provided by Gad Galili-Weizman Institute/Rehovot, Israel) conferring a Pnos-nptII gene (as a selectable marker) and a mutated *lysC* gene from *E. coli* (coding for a feed-back desensitised aspartatekinase III; Shaul & Galili, 1992) driven by the seed specific phaseolin promoter was also used (Figure 1). Single bacterial colonies containing each plasmid were grown separately in 25 ml LB liquid medium containing appropriate antibiotics overnight (16 h) at 28 °C on a shaker with 180 rpm to an OD_{650} of 0.8. The *Agrobacterium* culture was then diluted in a rate of 1:5 with B5 liquid medium amended with 0.5 and 1 mg/l kinetin and 2,4-D, respectively.

Transformation experiments

Two alternative *Agrobacterium*-mediated transformation protocols have been studied: protocol I based on *de novo* regeneration using thidiazuron TDZ (Riedel-deHaen), (Böttinger et al., 2001), and protocol II based on direct shoot organogenesis from meristematic cells of mature or immature embryo axes (Schroeder et al., 1993).

1. ***de novo* regeneration protocol:** Surface sterilized seeds of faba bean cultivars (Mythos, Giza 2, Giza 429, Giza 719 and Giza blanka) were soaked overnight in sterile water with shaking (90–95 rpm). The seeds were then germinated in darkness on $\frac{1}{2}$ MS-basal medium (pH 5.7). Etiolated internode segments or leaf explants of the plantlets 10 days after germination were used for co-cultivation with A*grobacterium*. Inoculation procedure, co-cultivation and regeneration was performed as

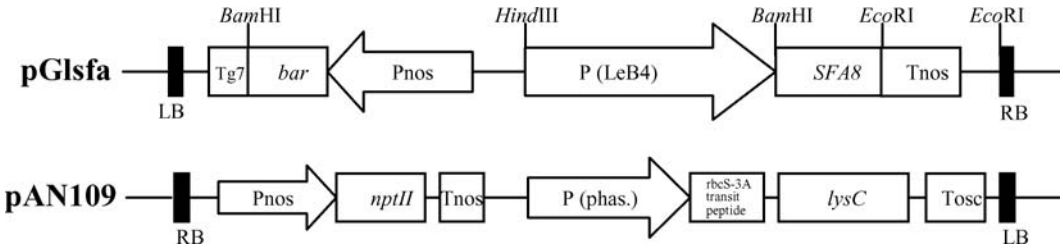

Figure 1. Schematic diagram of T-DNA region of plasmids pGlsfa and pAN109 (not drawn to scale).

described previously in Böttinger et al. (2001). Selection of the transgenic tissues was done by applying 2 mg/l PPT to the culture medium.

2. **Embryo axes transformation system:** Immature pods of the *Vicia faba* cultivars at 30 to 35 days after pollination were surface-sterilized with 70% (v/v) ethanol for 3 min and subsequently washed with sterilized distilled water 4–5 times before immature seeds were aseptically isolated. However, the surface-sterilized mature seeds of the cultivar 'Mythos' were soaked overnight in sterilized water under gentle agitation. The embryo axes of both mature and immature seeds were wounded by the removal of the root tips and slicing of the embryo axes to three or four segments longitudinally with a sharp blade wetted by the *Agrobacterium* suspension. Then, the explants were incubated in the *Agrobacterium* suspension for 15–20 min (immature embryos) and 30 min (mature embryos).

Agrobacterium inoculation procedure

About 30–40 wetted explants were co-cultivated with either EHA105/pGlsfa alone or with double infection of EHA105/pGlsfa and EHA101/pAN109 (they were mixed at a 1:1 ratio in the co-cultivation mixture) on solidified B5 medium with 0.5 mg/l kinetin and 1 mg/l 2,4-D at 25 °C in dark for 3–4 days. After co-cultivation, the explants were subsequently washed thoroughly with sterile distilled water and placed on solidified MS basal salt medium supplemented with B5 vitamins (Gamborg et al., 1968), 2 mg/l each of NAA and BAP and 150 mg/l Ticarcillin and 100 mg/l Combactam (Sulbactam-Na/ Pfizer) for 2 weeks.

Selection, regeneration and recovery of plants

The explants were subsequently transferred to regeneration medium containing MS basal salts, B5 vitamins, 4.5 mg/l BAP, 0.1 mg/l NAA, 100 mg/l Ticarcillin, 50 mg/l Combactam and 2 mg/l phosphinothricin (PPT) for selection. Every 2–3 weeks the cultures were passed to fresh medium for 6 months. The healthy resistant shoots were grafted onto etiolated seedlings of untransformed (wild type) *V. faba* (7–10 days-old) to recover mature plants. Grafting was done according to Pickardt et al. (1995).

PAT enzyme assays

PAT activity was analysed according to De Block et al. (1987) with modifications. Callus tissues or leaf explants (20–50 mg) were homogenized in a 1.5 ml Eppendorf tube with 40–100 μl ice cold extraction buffer (50 mM tris-HCl, pH 7.5, 2 mM Na-EDTA, 300 mg/l DTT, 300 mg/l bovine serum albumin (BSA) and 150 mg/l PMSF). After 15 min centrifugation at 4 °C and 14000 rpm, 15 μl of the protein extracts were mixed with 2 μg of PPT and 2 μl of ^{14}C-Acetyl-CoA (60 mCi/mmol). After 1 h incubation at 37 °C, 4 μl aliquots of the samples were spotted onto a silica gel thin-layer chromatographic plate. Ascending chromatography was carried out in a 3:2 (v/v) mixture of 1-propanol and NH4OH (25% NH3) for 2–3 h. Acetylated-PPT was visualized by autoradiography.

Assay of BASTA resistance

Transformants were tested in the greenhouse for the expression of the *bar* gene by painting the leaflets of the transgenic plants with BASTA (a commercial formulation of PPT containing 200 g/l ammonium glufosinate, Hoechst Ltd.) dilution at a concentration of 300–400 mg/l ammonium glufosinate. The opposite leaflet of each pair was marked and left untreated as a control. T1, T2 and T3 generations were tested by the same method or young plants (around 2–3 weeks after germination) were sprayed by the same BASTA solution. Resistance of leaflets or plants were scored after 7–10 days.

Southern blot analysis

DNA was isolated from young leaf tissues of faba bean using 'Plant DNeasy mini kit' (Qiagene). A 25 μg aliquot genomic DNA from transgenic and control plants were digested either with *Hind*III or *Bam*HI. Restriction reactions were done according to the manufacturer's instructions, with overnight incubation at 37 °C. The digested genomic DNA was electrophoresed through 0.8% (w/v) agarose gel, and transferred to positively charged nylon membrane (Boehringer/Germany). To show integration of the transgenes we hybridized the membranes with either a 750 bp *Eco*RI/*Bam*HI fragment containing the *SFA8* or a 1100 bp *bar* coding region from pGPTV-Gloc. plasmid (pGPTV-bar derivative, contains the *bar* gene and the β-glucanases gene) probes. The probes were

labelled using DIG high prime labelling kit (Boehringer Mannheim/Germany).

Polymerase Chain Reaction (PCR) analysis

Genomic DNA was isolated for PCR analyses according to the method of Sul & Korban (1996) from young leaves with minor modifications. The primers used for the amplification of a specific *SFA8* sequence (750 bp) were 5′-ACT AGT ATG GCA AGG TTT TCG ATC-3′ and 5′-GAG CTC TTA CAT TTG GCA TGG TTG-3′. The primers were used in a concentration of 30 pM of each primer with 0.5 μg/genomic DNA, 1.5 mM $MgCl_2$, 2 units of Taq polymerase (Appligene) and 80 μM dNTP (each). The reactions were run for 30 cycles for 1 min denaturation at 94 °C, annealing at 57 °C for 1 min and extension for 2 min at 74 °C and finally a 10 min extension step at 74 °C was included.

RNA isolation and reverse transcriptase (RT)-PCR analysis

The transformed lines were analyzed by RT-PCR to characterize the expression of *SFA8* gene and legumin gene (as an internal control) at RNA level. Total RNA was isolated as described previously in Böttinger et al. (2001). The first strand cDNA was constructed by Expand Reverse Transcriptase kit according to the manufacturer's instruction (Roche/Germany) using oligo$(dT)_{18}$ primers. The following primers were used for the amplification of the legumin transcript (1200 bp): Leq1: 5′-TCC AGA GCT CCA CAG TCA CAA TGT $\overline{\text{CCA}}$ AAC-′3 + Leq2: 5′-TGC ACA GCT GTT GCA CTC CTT AGC $\overline{\text{ATG}}$ ATC-′3. Specific *SFA8* Primers and PCR condition were the same as mentioned above.

Western blot analysis

Total soluble seed proteins were extracted from either mature or immature seeds (30 days after pollination) of clones transformed with the pGlsfa plasmid. 20 mg seed flour or 200 mg immature cotyledons were homogenized in 200 μl extraction buffer. For SFA8 protein the following extraction buffer was used: 25 mM Tris (pH 9.0), 0.5% SDS and 10% Glycerol. 40–80 μg protein were separated in a 15% SDS polyacrylamide gel (Laemmli, 1970). The separated polypeptides were blotted onto PVDF membrane (BioRad). The SFA8 protein was identified using rabbit anti-sunflower 2S albumin-specific polyclonal antibody. For detection, a peroxidase-based Vectastain detection kit (ABC kit, Vector Laboratories, USA) was used according to the manufacturer's instructions. Total soluble sunflower seed protein (20–30 μg) was used as a positive control.

Results

Genetic transformation and recovering of the transgenic plants

Control experiments showed that PPT at 2 mg/l totally suppressed callus development from wild type faba bean explants (epicotyl segments and leaf explants) cultured on MS-medium supplemented with 0.5 mg/l of 5-benzylaminopurine (BAP), NAA and 2,4-D. This PPT concentration was sufficient to suppress shoot development from the embryo axes grown on MS medium supplemented with 4.5 mg/l BAP as well. All the control explants died within 3–4 weeks. The feasibility of the embryo axes transformation strategy developed in this experiment was initially evaluated by monitoring the number of regenerated shoots from the embryo axes explants cultured on medium with high BAP concentration (4.5 mg/l) where routinely 4–5 shoots regenerated from each explant.

In series of transformation experiments with 6 cultivars of faba bean i.e. Mythos, Albatross, Giza 2, Giza 429 Giza 716 and Giza blanka, the explants (immature or mature embryonic axes) were inoculated with *Agrobacterium* strain EHA105/pGlsfa (harbouring *SFA8* and *bar* genes) alone or co-transformed with EHA101/pAN109 which contains a mutated *lysC* gene from *E. coli* and *npt II*. A total of 1967 embryo axis explants from total 6 cultivars were co-cultivated with the *Agrobacterium* strains. In the co-transformation experiments, selection was done by one of the selectable markers (2 mg/l PPT) and the transformed plants were screened for the presence of the genes derived from the second plasmid. After 3–4 weeks of culturing on selective medium, all control explants had died. On the other hand, a number of *Agrobacterium*-treated explants started to regenerate (*via* organogenesis) and about 3–4 shoots appeared from each explant on the regeneration medium. The shoots selected for 4–6 months were grafted *in vitro* and finally transferred to the greenhouse to set seeds (T1). A total of 7 stable independent transformants (*SFA8*) from 2 cultivars (Mythos and Albatross) have been recovered. Regarding the co-transformation experiments, transformed

Table 1. Transformation efficiency in different *Vicia faba* cultivars using embryo axes

Cultivar	Explants type	Plasmids	Number of explants treated	Number of putative (T0) transformed lines (SFA8)	Number of stable transformed lines (SFA8)	Transformation efficiency % (SFA8)
Mythos	Immature embryo axes	pGlsfa	141	8	1	0.71
		pGlsfa and pAN 109	100	7	2	2.00
	Mature embryo axes	pGlsfa	220	11	1	0.45
Albatross	Immature embryo axes	pGlsfa	667	11	1	0.15
		pGlsfa and pAN 109	380	3	2	0.53
Giza 2	Immature embryo axes	pGlsfa	81	2	0	0
Giza 429	Immature embryo axes	pGlsfa	132	0	0	0
Giza 716	Immature embryo axes	pGlsfa	123	3	0	0
Giza blanka	Immature embryo axes	pGlsfa	123	0	0	0

plants (T1) were screened for the presence of both the T-DNAs by PCR analysis. It was found that only the T-DNA encoding the selectable marker PPT has been integrated in the transgenic plants (see below). The time needed to obtain T1 seeds by this protocol is about 9–10 months. Table 1 presents a summary of the results, which were obtained with the embryo axes transformation system.

On the other hand, the *de novo* transformation protocol has been tested in the present study with different agronomically important cultivars, e.g. the German cultivars 'Mythos' and four Egyptian cultivars namely Giza 2, Giza 429, Giza 716 and Giza blanka. Callus clones were selected from two different types of explants (leaf and stem explants) of all cultivars on PPT containing medium. The callus proliferation differed from cultivar to cultivar and from one type of explant to the other (data not shown). The frequencies of callus induction under selective conditions were between 4.30–31.59%. Shoot bud regeneration from the selected calli was 6.6% with Giza 716, 3.7% with Mythos, 2% with Giza 2, 0.9% with Giza 429 and 0% with Giza blanka. Regeneration capacity from the selected callus was very low, in contrast to model plants.

PAT assay and leaf paint

PAT activity assays of extracts of T1 progeny of plants selected on media containing PPT confirmed that the *bar* gene was active (Figure 2A). The T1 plants were analyzed by testing the expression of the *bar* gene in the greenhouse by applying the leaf painting assay or spraying the young plants with the same dilution of the herbicide. Within 2 days necrotic spots appeared on the untransformed leaves. Ten days after BASTA application, the treated transgenic plants and leaflets showed complete tolerance, in contrast to non-transformed plants showed leaflets, which were completely necrotic (Figure 2B–2C).

Southern blot and PCR analysis

Southern blot analyses were performed to confirm the integration of the transgenes. Genomic DNA of primary transformant of clone G2/E1/1/1 (cv. Giza 2, recovered from embryo axes transformation) was digested with *HindIII*, which cleaves the pGlsfa vector only once, transferred to nylon membranes, and hybridised with the DIG labelled *bar* gene (Figure 3A). The hybridisation profile exhibits integration pattern consistent with 2 copies of the gene. Inheritance of the transgene was confirmed also by Southern analysis. Plant genomic DNA was isolated form young leaves of the transgenic progeny plants of different transgenic clones. DNA digestion was performed either with *BamHI* or *HindIII* and the fragments were subjected to Southern-blot analysis using either 1.1 kb *bar* or *SFA8* (750 bp) probes. Figure 3B shows the hybridisation blot of *HindIII* digested genomic DNA (T1 generation of lines G2/1/2 cv. Giza 2 and ME1/2/1 cv. Mythos) after hybridization with a DIG-labelled 750 bp *SFA8*-fragment which hybridises with the T-DNA/plant-DNA junction fragments. Line ME1/2/1 contains a single copy of integrated T-DNA, whereas line G2/1/2 (recovered from *de novo* regeneration protocol) is harbouring two copies of T-DNA; no hybridisation signal was observed in the WT. Digestion of the genomic

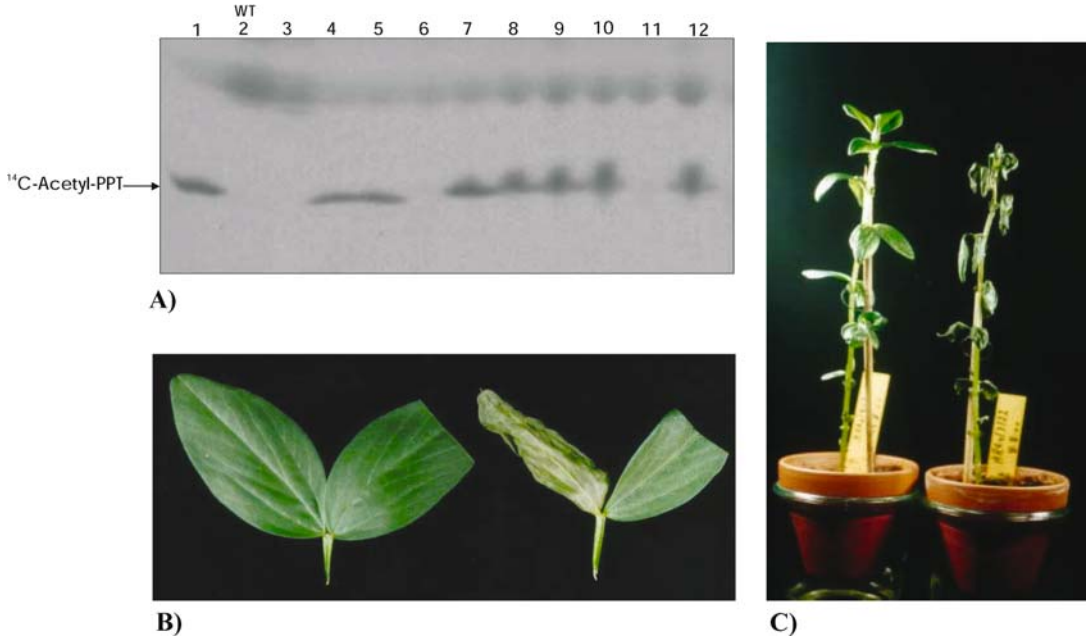

Figure 2. A) Expression of the *bar* gene by assaying the PAT activity in stable transgenic plants (T1) cv. 'Mythos' regenerated form embryo axes transformation of two transgenic clones; MfkE1/2/2 (lanes from 3–6) and clone ME1/2/1 (lanes from 7–12), lane 2 is untransformed plant (WT) and lane one is a positive control, transgenic faba bean expressing *bar* gene. B) herbicide leaf painting test showing the resistance of transgenic leaf to BASTA application (left). C) resistance of transgenic plants to spraying with BASTA (300–400 mg/l ammonium glufosinate); the control non-transgenic plant died after spraying (right).

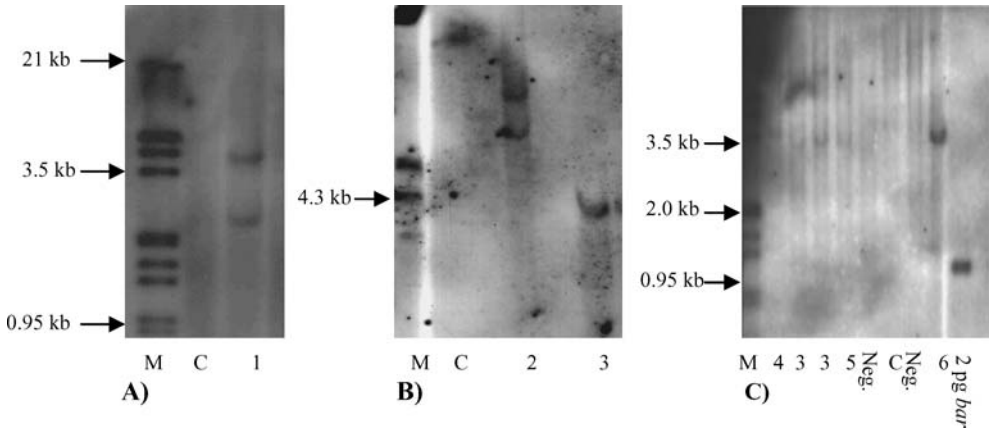

Figure 3. Southern blot analysis of representative transgenic plants **A)** 25 μg *HindIII* digested genomic DNA, isolated from T0 plant of clone G2/E1/1/1 (cv. Giza 2), blotted to a nylon membrane and probed with the DIG labelled 1.1 kb *bar* gene. **B and C)** The corresponding Southern blot analysis for *SFA8* of clones G2/1/2 (cv. Giza 2) and ME1/2/1 cv. Mythos (B) and for the *bar* gene of cv. Mythos clones Mfka/1, and two descendants of ME1/2/1, MfkE1/2/2 and M/7/85 (C) of the transgenic T1 progenies, genomic DNA was digested with either *HindIII* or *BamHI* respectively.

DNA with *BamHI* that cleaves the vector pGlsfa twice, should prove the integration of the *bar* gene and the full-length of legumin promoter in the transformants. In all samples which were analysed, it could be found that at least one single copy of *bar* gene was integrated into the plant genome. The expected hybridising band of 3.5 kb is seen in Figure 3C. Because of the enormous genome size of *Vicia faba* (13059 Mbp/1C, Bennett & Smith, 1976), hybridisation signals of single genes are very faint and hard to detect. The inheritance of the

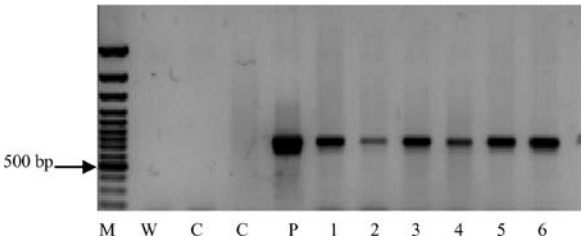

Figure 4. PCR analysis of SFA8-transgenic faba bean cv. Albatross (T1 progeny, recovered from embryo axes transformation) of clones Abk1/5/1 (lanes 1–4) and Abk3/4/3 (lanes 5 and 6)*: M* Molecular weight marker 100 bp, *W* water, *C* non-transformed control plants, *P* plasmid pGlsfa. The size of the amplified fragment is 750 bp.

foreign genes in G2/E1/1/1 clone has not been confirmed in the next generation.

PCR analysis the transgenic plants confirmed the integration of the transgene. All the clones tested showed the expected band of 750 bp for the *SFA8* gene (Figure 4), while no fragment was amplified in untransformed control DNA samples (WT). Inheritance of the transgenes was confirmed by PCR until T3.

Sunflower 2S albumin (SFA8) expression

The expression of the *SFA8* transgene in transformed faba bean clones was studied by RT-PCR and western blot analysis. RT-PCR analysis with total RNA samples from individual immature embryos (30 days after pollination) of independent transgenic clones in T2 or T3 generation showed the accumulation of the expected *SFA8* transcript. No transcript signal was observed in untransformed control plants (WT) or negative plants. In addition, RT-PCR of the legumin B4 gene was used as an internal control (Figure 5A). RT-PCR of *SFA8* results in the amplification of a fragment of 550 bp, lacking the intron sequence of 200 bp. The legumin transcript was used as a control for cDNA synthesis and PCR reaction and showing a strong signal in all samples tested. The RT-PCR amplified legumin fragment has a length of 1200 bp.

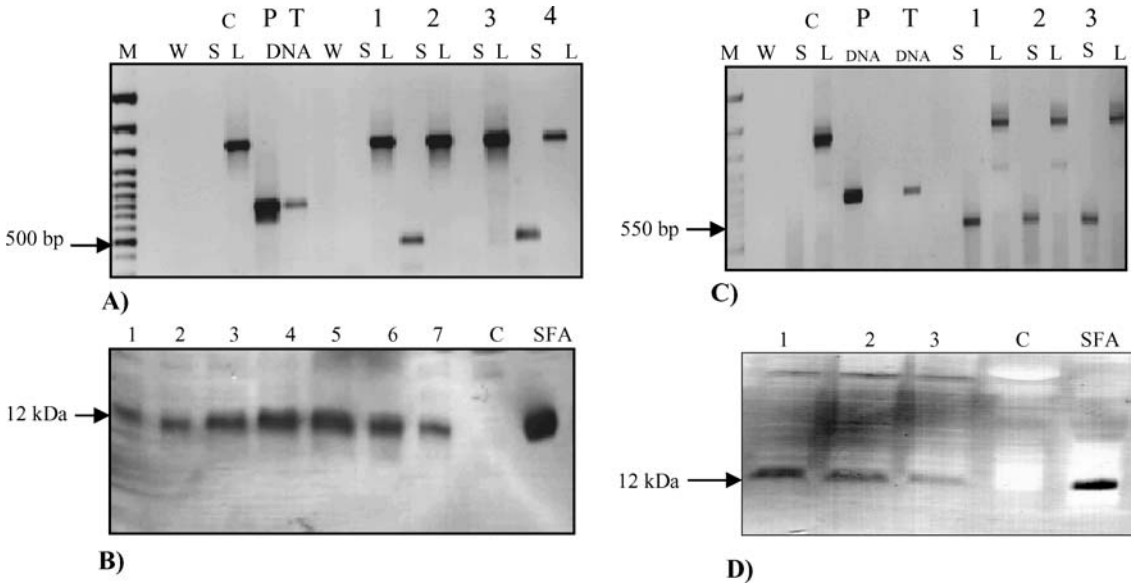

Figure 5. (A–D) Molecular analysis of transgenic faba bean cvs. Mythos (A–B) and Albatross (C–D) transformed with pGlsfa plasmid. **A)** RT-PCR analysis of *SFA8* (S lanes) and Legumin (L lanes) transcription in the immature embryos of individual seeds of independent transgenic clones. *SFA8* transcript resulted in the amplification of 550 bp fragment, lacking the intron sequence of 200 bp. Legumin transcript resulted in the amplifications of 1200 bp in all samples tested. **B)** Western blot showing the accumulation of the SFA8 protein (12 kDa) in the T2 transformant of plant Mfka/1.3.2 (lane 7) and plant Mfka/1.3.1 (lane 6) and its progeny (T3) lanes 1–5. Each lane was loaded with 80 μg of total extractable protein from mature dry seeds and 40 μg of total extractable seed protein of sunflower was used a positive control. **C)** RT-PCR analysis for detecting *SAF8* (S lanes) and legumin (L lanes) mRNA accumulation in cv. Albatross transformants. The *arrow* indicates the amplified fragment of 550 bp. **D)** The corresponding western blot analysis of individual immature seeds of Albatross transgenic plant. 40 μg total SDS-seed protein were loaded onto each lane. As a positive control 26 μg of total extractable seed protein of sunflower was loaded (SFA lane). The *arrow* indicates the expected 12 kDa SFA8 band. *M* Molecular Marker, *W* Water, *P* pGlsfa plasmid, *C* non-transformed control plant. *T* genomic DNA from transgenic faba bean with *SFA8* gene.

The accumulation of the sunflower 2S albumin protein in the mature and immature seeds of the transgenic *Vicia faba* plants was determined by western blot. The western blot analysis revealed that the SFA8 protein was expressed and accumulated in most of the transgenic faba bean clones, as judged from the presence of the 12-kD protein (Figure 5B). No signals were observed in untransformed faba bean (WT) or in the negative plants. In all cases where both RNA and protein were detectable, the presence of the SFA8 protein was associated with the corresponding mRNA. The stability of SFA8 protein through different generations was analysed by western blot. Figure 5B shows that the SFA8 protein was accumulated in plant Mfka/1.3.1 (T2) and its progeny (T3) at a uniform level. With regard to the transgenic clones derived from cv. 'Albatross', the *SFA8* gene expression and its respective protein accumulation were monitored by RT-PCR and western blot analysis. Figures 5C and 5D represent the results of *SFA8* transcript detection by RT-PCR and the corresponding Western blot analysis in the cotyledons of immature embryos (30 days after pollination) of T2 seeds of clone AB1/2/3.

Discussion

In the present study we report here the production of transgenic faba bean containing the *SFA8* gene from sunflower and *bar* gene as a selectable marker. To our knowledge, the first time the *bar* gene from *Streptomyces hygroscopicus* has been used for faba bean transformation and it is also the first time that transgenic Albatross and Giza 2 cultivars were recovered. Regeneration of fertile transgenic faba bean cv. Mythos by *Agrobacterium*-mediated transformation has been reported by Böttinger et al. (2001). Their approach was based on plant regeneration from internodal stem segments derived-callus (meristem-lacking tissues). The low regeneration efficiency in our experiments is in agreement with the previous work done by Böttinger et al. (2001) where regeneration frequencies were between 2.7% and 6%, or 6% in untransformed callus from Tegeder et al. (1995) with *Vicia faba*. The recovery of seed producing putative transformed plants under these circumstances took about 16–24 months, which is a considerably long period. The *de novo* transformation experiments were conducted as a control for comparison with the embryo axes transformation system. The main constraints in this protocol were the poor regeneration ability following a callus phase, the reduced fertility and the high percentage of phenotypic abnormalities in the regenerated plants. This can be ascribed to the fact that explants and callus cells of *Vicia faba* tend to produce high amounts of phenolic compounds resulting in subsequent toxification of the tissue (Bieri et al., 1984; Selva et al., 1989) and also is possibly due to the long cultivation time *in vitro* (around 7–16 months, McClintock, 1984).

Transformation protocols based on *de novo* regeneration of shoots from callus tissues have rarely been applied to produce transgenic grain legumes. For example Puonti-Kaerlas et al. (1990) obtained transgenic pea (Pisum sativum) by regeneration of transformed shoots from hygromycin resistant callus derived from epicotyls and shoot cultures. This process took 15 months until the primary transformed plants produced seeds. In 1992 they reported chromosome doubling (tetraploidy) in their transgenic pea. There are also successful transformation systems which are based on the *de novo* regeneration protocol: *Vicia narbonensis* (Pickardt et al., 1995), *Arachis hypogaea* (Cheng et al., 1996) and with *Phaseolus acutifolius* (Dillen et al., 1997). This confirms the fact that the major limitation of grain legumes transformation in general is the requirement for effective *in vitro* regeneration system. This has strongly limited the application of genetic engineering techniques to improve this important group of plants.

Transformation strategies which minimize the *in vitro* culture period and avoiding the callus phase would therefore be advantageous. The major success in legume transformation was achieved by methods based on transformation of the pre-existing meristems on the embryo axes, cotyledonary nodes, shoot tips or nodal explants. This approach has been successfully applied in combination with *Agrobacterium* in different grain legumes such as pea (Schroeder et al., 1993; Bean et al., 1997), chickpea (Krishnamurthy et al., 2000; Kiesecker, 2000), *Lupinus angusitifolius* (Pigeaire et al., 1997) and soybean (Yan et al., 2000; Olhoft et al., 2003). In combination with direct gene transfer systems transformation success was reported from soybean (McCabe et al., 1988; Russell et al., 1993; Sato et al., 1993) peanut (Brar et al., 1994), *Phaseolus vulgaris* (Russell et al., 1993; Aragao et al., 1996).

In the present study, the potential of *Agrobacterium* mediation for *Vicia faba via* meristematic cells was proved and optimised. The mature and immature embryonic axes of six elite faba bean cultivars (i.e. Mythos, Albatross, Giza 2, Giza 716, Giza 429 and Giza blanka) were used as explant sources. Regeneration was successfully applied for the production of

seven transgenic faba bean clones from Mythos and Albatross cultivars. The transferred genes were shown to be stably inherited and expressed in the next progenies of transformed plants till T4 (data not shown). Transformation frequencies were ranging from 0.15% to 2.0%.

The actual transformation system developed here overcomes many of the difficulties which previously were reported by Böttinger et al. (2001). The use of embryonic axes which were cultivated on media containing high concentrations of cytokinins in combination with low auxin concentrations enhanced the direct shooting without an intermediate callus phase. By this method, the possibility of somaclonal variation has been reduced to a minimal level. This transformation system was an adaptation of the protocol which previously was reported by Schroeder et al. (1993) for *Pisum sativum*. The developed transformation system allowed obtaining the primary seeds bearing transformants (T1) to be recovered within approximately 9–10 months.

The comparably high frequency of plants which did not show the transfer of the introduced genes to the next generations was possibly due to chimeric events leading to T0 plants (Table 1). Our objective was to ascertain PPT suitability for differential growth between transformed and non-transformed tissues in faba bean. The relatively low concentration of PPT (2 mg/l) which used in this study might favor the cells reaching a critical size, which supposed to be necessary for shoot development and increased the probability of obtaining transgenic plants. However, the application of high concentration of PPT may lead to widespread cell death that probably affects the viability of transgenic cells and interferes with successful development of transgenic shoots. Notably, short-term selection in principle increases the possibility for chimeric plants, which are mainly useless. Thus, it can be recommended that the regenerated shoots should be selected for at least 6 months before recovering the plants by micro-grafting. The recovery of both clonal and chimeric primary transformants were repeatedly reported in many studies which applied meristematic cell transformation either by biolistic approach (Christou & McCabe, 1992; Sato et al., 1993) or with *Agrobacterium*-mediated transformation (Bean et al., 1997). If this is the case, chimeric transformants are not necessarily limiting the value of this transformation method. Those chimeric individuals, which are germ-line (L2)-transformants gave rise to clonally transformed progeny.

One of the aims of this study was the modification of the seed storage protein composition of *Vicia faba* in order to enhance the sulphur containing amino acids (methionine and cysteine) by means of genetic transformation. The coding sequence of the methionine-rich 2S albumin gene from sunflower *SFA8* (Kortt et al., 1991) was transferred to faba bean. The expression of the *SFA8* gene was studied by detection of the transcript in the immature cotyledons of transgenic faba bean by RT-PCR and by studying the accumulation of the foreign protein (Western blot). In order to promote a seed specific expression, the coding sequences of the *SFA8* gene was driven by the LeguminB4 promoter (Bäumlein et al., 1987, 1988). The usage of such promoter for seed specific expression of the foreign genes like the Brazil nut 2S albumin gene was reported previously by Pickardt et al. (1995) and Saalbach et al. (1995a, 1995b). RT-PCR was used to amplify the transcripts of the *SFA8* gene and the legumin gene as an internal control. The mature protein of sunflower seed albumin (SFA8) consists of a signal polypeptide chain of 103 amino acids with a molecular weight of 12.133 Da (Kortt et al., 1991). The accumulation of the SFA8 was detected by western blots in five transgenic faba clones with relatively strong signals. This implies that the legumin promoter (LeB4) controls the gene expression as expected in a seed specific manner. These results are in accordance with those of the other reports of Pickardt et al. (1995) and Saalbach et al. (1995a, 1995b). Due to the lack of purified sunflower 2S albumin, it was difficult to estimate the accurate amount of the foreign protein in the transgenic faba bean.

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