Development of an efficient transformation system to field bean (*Vicia faba*)

Manipulation of the sulphur-rich protein content *via* genetic engineering

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Zusammenfassung

Das Hauptziel der vorliegenden Dissertation ist es, die Zusammensetzung der Speicherproteine in Samen der Ackerbohne durch die Einführung von Fremdgenen so zu verändern, dass ein möglichst hoher Anteil an proteingebundenem Methionin gebildet wird. Als Fremdgene dienen heterologe DNA-Sequenzen, die für methioninreiche Proteine kodieren. In dem vorliegenden Projekt wurde ein 2S-Albumingen aus *Helianthus annuus* (*SFA8*, Kortt et al. 1991) unter Kontrolle eines samenspezifischen Promoters in die Ackerbohne übergetragen.

Die Transformation der Ackerbohne ist schwierig, das einzige bislang existierende Protokoll wurde von Böttinger et al. (2001) veröffentlicht. Das Verfahren beruht auf einer *de novo*-Bildung von Sproßinitialen aus dedifferenzierten Zellen. Die Effizienz dieser Methode ist allerdings sehr gering, und ein weiterer Nachteil liegt darin, daß das Protokoll einen relativ großen Zeitraum erfordert (ca. 16-20 Monate bis zur Samenreife der Primärtransformanten). Als zweite Methode wurde in der vorliegenden Arbeit ein Verfahren verwendet, welches die natürlicherweise vorhandene Fähigkeit von Sproßmeristemen nutzt, unter Einfluß von Cytokininen durch Ausbildung von Seitensprossen *in vitro* zu proliferieren. Mit beiden Methoden ließen sich eine Reihe von transgenen Pflanzen von drei Elite Sorten erzeugen. In dem Zusammenhang wurde erstmals einen *Agrobacterium*-vermittelter Gen-Transfersystem in Sproßmeristemen bei *Vicia faba* entwickelt und als Selektionsmarker das *bar*-gen wurde genutzt. Die Integration der fremden Gensequenzen konnte durch genomische Analysen (Southern Blot, PCR), sowie durch Expressionsstudien (Aktivität des Selektionsmarkergens) und *SFA8* gen (RT-PCR, Western blot und Aminosäureanalysen reifer Samen) bis zur T3 Generation nachgewiesen werden.

Stichworte:

***Vicia faba*; *Agrobacterium tumefaciens*; Schwefelreiche Proteine.**

Abstract

The main goal of the present research is the enhancement of the sulphur containing amino acids in the seed storage proteins of *Vicia faba* through the introduction of foreign genes, i.e. a gene coding for a sulphur rich sunflower albumin (SFA8/Kortt et al. 1991) into the faba genome. The coding sequences of the *SFA8* gene was driven by a *Vicia faba* legumin B4 promoter which elicits seed-specific expression. *Vicia faba* is one of the most recalcitrant species for *in vitro* manipulation and transformation. The only published *Vicia faba* transformation protocol was reported by Böttinger et al. (2001). This protocol was based on the *de novo* regeneration from dedifferentiated cells (calli). This process is time consuming and of relatively low efficiency (16-20 months until transgenic plants bearing seeds). In the present study a second *Agrobacterium*-mediated transformation system based upon direct shoot organogenesis after transformation of meristematic cells (derived from mature and immature embryo axes) was developed in combination with the *bar* gene as a selectable marker. Independent transgenic plants from three cultivars were recovered from both transformation systems. The integration of the foreign genes was confirmed by molecular analysis (Southern blot and PCR), also the expression of the marker gene (*bar*) was studied by either PAT-assay or Leaf Paint Test. SFA8 gene expression was analysed by RT-PCR, western blot and by amino acid analysis in the seed meal of the transgenic seeds up to the T3 generation.

Keywords:
***Vicia faba*; *Agrobacterium tumefaciens*; Sulphur-rich protein.**

Contents

Abbreviations

2,4-D	2,4-dichlorophenxy acetic acid
APS	Ammonium persulphate
BA	6-Benzyladenine
BAP	6-Benzylaminopurin
bp	base pair
BPB	bromo-phenol blue
BSA	Bovine Serum Albumin (fatty acid free)
CTAB	Hexadecyl Tri-Methylammonium
DAB	Diaminobenzidine tetrahydrochloride
DMSO	Dimethylsulfoxid
EDTA	Ethylendiamintetraacetate
GA	Gibberellic Acid
GUS	β-glucuronidase
kb	Kilobase
NAA	1-Naphthaleneacetic acid
PCR	Polymerase Chain Reaction
PPT	Phosphinothricin
PVP	Polyvinylpyrrolidone
SDS	Sodiumdodecylsulfate
TDZ	Thidiazuron
X-Gluc.	5-bromo-4-chloro-3-indolyl glucuronide

1. Introduction and review of literature

1.1 General introduction

Legumes belong to one of the largest families of dicotyledons, *Leguminosae* (*Fabaceae*). They are very important for their value as food, feed and for their role in the biological fixation of aerial nitrogen (Duke 1981). The genus *Vicia* comprises approximately 150 species of the family *Leguminosae* distributed in temperate and subtropical areas of the world (Maxted et al. 1991, Maxted 1993). The economically most important species of this genus is the faba-bean (*Vicia faba* L.), also referred to as broad bean, horse bean or field bean. This species alone occupies nearly $3.2x10^6$ ha worldwide in 1991 (FAO statistics, 1992) with a world production of 3.256 million tons in 2000. Even though faba bean has been cultivated in many countries, 49% of the total production comes from China followed by Egypt (10,8%), Ethiopia (8.5%), and Australia by 5.8% in 2000 (FAO, statistics 2001).

Unfortunately, its susceptibility to environmental conditions, biotic stress and instability of the yield made this crop less attractive. Also, difficulties in pollination control and the limited genetic pool has led to slow down progress in varietal improvement (Bond 1987). Today, breeding programs of *Vicia faba* could be supplemented by recombinant DNA-technology, which requires, however, the development of reproducible protocols for *in vitro* manipulation of single cells or explant tissue and the subsequent regeneration of plants. *Vicia faba* exhibits, like most other grain legumes, a rather low amenability to tissue culture conditions, mainly due to difficulties in the regeneration of shoots or somatic embryos from dedifferentiated tissue.

1.2 *In vitro* regeneration of *Vicia* species

1.2.1 Regeneration from explant tissue

Vicia species have been subjected to cell- and tissue culture experiments since approx. 40 years. The first attempts to cultivate *Vicia faba in vitro* focused on the

optimal growth of callus tissue or suspension cultures rather than the induction of shoot morphogenesis and plant regeneration (Venketeswaran 1962, Grant and Fuller 1968, Mitchell and Gildow 1975). The influence of media composition and explant source on the initiation and maintenance of cultures were tested and the conditions optimized for a maximum increase in callus fresh weight. Poor growth rates and an increase of necrotic tissue during cultivation were described. Suspension cultures could also be established in *Vicia hajastana* (Singh et al. 1972) which served as source for protoplast isolation in later studies (Kao et al. 1974, see below). Since *Vicia faba* is an excellent karyological object, studies on callus growth were also performed by Cionini et al. (1978) and Jalaska et al. (1981) to investigate the pattern of chromosomal instability during callus development. The low morphogenic potential of *Vicia faba* cells cultured *in vitro* was first mentioned by Röper (1979). With the aim to develop a system for plant regeneration from single cells, he established callus and cell suspension cultures over a long period of time, but all attempts to initiate shoot regeneration remained unsuccessful.

During the following decade a number of reports were published describing the cultivation of tissues containing shoot apical meristems and the subsequent recovery of shoots (Martin et al. 1979, Cheyne and Dale 1980, Galzy and Hamoui 1981, Schulze et al. 1985, Busse 1986, Fakhrai et al. 1989, Selva et al. 1989, Taha and Francis 1990). Excised apical meristems, nodal buds and cotyledonary nodes were exposed to media containing cytokinins (in most cases BA) alone or in combination with low amounts of an auxin. Under these conditions shoots preferentially develop from preexisting meristems. Since cytokinins are effective in removing apical dominance (Skoog and Schmitz 1972) this treatment continuously promotes the development of young meristems of the apical dome(s) (Steves and Sussex 1989) to lateral buds and shoots, which are again themselves suppressed in their further growth. This cycle of simultaneous induction and suppression finally gives rise to multiple bud/shoot proliferation. Similar protocols have repeatedly been described for many other grain legumes (e.g. Cheng et al. 1980, Kartha et al. 1981, Martins 1983, Griga et al. 1986, Jackson and Hobbs 1990, Malik and Saxena 1992a/1992b, Brandt and Hess 1994). The origin of shoots (axillary vs. adventitious) in these regeneration systems has frequently been discussed and investigated in histological studies. The development of shoot initials from superficial layers and the absence of a vascular

connection to axillary buds are generally taken as evidence for a *de novo* formation of shoots (e.g. Fakhrai et al. 1989). Since both attributes apply also for the young shoot meristems of the apical dome, these observations do not provide evidence for an adventitious origin of shoots. However, even if the distinction between *de novo* organogenesis and shoot proliferation from preexisting meristems is not trivial, this question may finally be less relevant, considering the development of transformation systems as the main purpose of these studies.

Today several transformation protocols in grain legumes are based on the BA-induced shoot development from embryo axes and cotyledonary nodes, using *Agrobacterium tumefaciens* (e.g. Bean et al. 1997/pea, Sato et al. 1993/soybean) or the biolistic approach (McCabe et al. 1988/soybean; Brar et al. 1994/peanut; Russel et al. 1993, Aragao et al. 1996/common bean). The recovery of both, clonal as well as chimeric primary transformants were repeatedly described in these studies (e.g. Christou and McCabe 1992, Sato et al. 1993), indicating the occurrence of single- and multiple cell origin of shoots. Chimeric transformants do not necessarily limit the value of these systems: A number of chimeric individuals are germline (L2)-transformants giving rise to clonal transformants in the progeny.

A serious constraint in *Vicia faba* tissue culture is the deterioration of explant material and cultivated tissue as a result of the action of phenolic compounds. Bieri et al. (1984) and Selva et al. (1989) examined the effect of various chemical and physical parameters in axillary shoot cultures. In their studies low temperatures were found to limit the formation of phenolics.

Plantlet regeneration from explants lacking preexisting shoot meristems was claimed by Thynn and Werner (1987). Callus was initiated from epicotyl segments on B5-basal medium (Gamborg et al. 1968) supplemented with 0.2 mg/l NAA, and shoot development was achieved on a subsequent transfer to B5-medium containing 0.05 mg/l NAA and 0.5 mg/l kinetin. With the exception of the study of Tegeder et al. (1995) on protoplast regeneration (see below) this is the only report of shoot regeneration from an explant without apical or axillary shoot meristems in *Vicia faba*.

Somatic embryogenesis in callus and suspension cultures derived from immature cotyledons of *Vicia faba* was reported by Griga et al. (1987). They followed the traditional pathway of somatic embryogenesis induction: 2,4-D initiation of callus and subsequent lowering or removal of 2,4-D (Ammirato 1983). The formation of bipolar structures were observed. These structures did obviously not contain shoot meristems, as only root development occurred on further cultivation. Somatic embryogenesis was also described for *Vicia narbonensis* in two independent reports. In a study of Albrecht and Kohlenbach (1989) leaf-derived callus was cultured for of 5-6 months in a series of different MS-media supplemented with combinations of picloram/BA, 2,4-D/BA and 2,4-D/Kinetin. Somatic embryo development resulted from a last step on hormonefree MS-medium. Further development to plants was not reported. Pickardt et al. (1989) described a protocol in which shoot tips from young seedlings cultivated on MS-medium containing 0.1-10 mg/l 2,4-D gave rise to callus which formed somatic embryos if 2,4-D was removed and replaced by 1 mg NAA/l in a subsequent cultivation step. Plantlets derived from somatic embryos could be grown to maturity. In this regeneration system the frequency of explants producing embryogenic callus as well as the mean number of embryos per embryogenic callus increased with the 2,4-D concentration initially used for callus induction. Later studies of Pickardt et al. demonstrated that shoot tips could be replaced by epicotyl- and stem segments (unpublished data, 1990). An improved protocol was subsequently combined with the *Agrobacterium tumefaciens*-mediated gene transfer (Pickardt et al. 1991, see below).

The results of Albrecht and Kohlenbach and of Pickardt et al. revealed that, at least in *Vicia narbonensis*, cells of a differentiated tissue (lacking apical or axillary meristems) like leaf sections or stem segments can be reconverted to an embryogenic state. Compared to *Vicia faba*, where by that time regeneration of plants had occurred most probably only from preexisting shoot meristems, the *in vitro* cultured cells of the less domesticated *Vicia narbonensis* obviously possess a higher morphogenic potential.

1.2.2 Regeneration from protoplast

Vicia hajastana protoplasts were isolated from suspension cultures in studies on protoplast fusion (Kao and Michayluk 1974, Kao et al. 1974) and in order to evaluate the nutritional requirements for growth of protoplasts at a low population density (Kao and Michayluk 1975). In these studies division of protoplast and callus formation was achieved. Donn (1978) described the isolation of protoplasts from leaves in *Vicia narbonensis*. A 7-day preculture of leaves on MS-medium containing 0.5 mg/l of each BA and p-chlorophenoxyacetic acid appeared to be crucial to obtain cell division and callus formation. The addition of asparagine, glutamine, and serine enhanced the rate of division. Attempts to initiate shoot regeneration in protoplast derived calli were unsuccessful. Only root formation occurred in a low frequency on a medium supplemented with 0.1 mg/l BA and 0.1-0.5 mg/l p-chlorophenoxyacetic.

Binding and Nehls (1978a, 1978b) isolated protoplasts from leaves and shoot apices of *Vicia faba*. Division of protoplasts could be initiated and maintained in KM-medium or a combination of V-47 medium (Binding 1974) and KM-medium containing 0.5 mg/l BA, 1 mg/l NAA and 0.1 mg/l 2,4-D. Highest plating efficiencies were achieved if less than $3x10^3$ protoplasts per ml of *Vicia faba* were cocultured with $0.5\text{-}10x10^4$ protoplasts of *Petunia hybrida*. The authors suggest that the coculture effect is probably congruent to a feeding layer sytem (Binding and Nehls 1978a). In further experiments on somatic cell hybridisation between *Vicia faba* and *Petunia hybrida* the same authors obtained three hybrid clones, one of them could be propagated at least for 9 months. The fusion hybrids contained predominantly nuclei or chromosomes of one or the other species and a few chromosomes of the second parent (Binding and Nehls 1978b). In both studies initiation of shoot or root morphogenesis was not reported. Protoplasts were also isolated from suspension cells of *Vicia faba* (Röper 1981). In KM-medium supplemented with 0.5 mg/l BA, 0.2 mg/l 2,4-D and 0.5 mg/l IAA protoplasts divided and formed cell colonies that gave rise to proliferating calli. Regeneration of shoots was not reported.

During the next 14 years no reports on protoplast regeneration in *Vicia* species were published. Tegeder et al. (1995) demonstrated for the first time the recovery of mature plants from protoplasts of *Vicia faba*. Protoplasts were isolated from shoot

tips of etiolated seedlings and embedded in alginate discs. In a screening of 10 cultivars of *Vicia faba* plating efficiencies, survival rates, and the regenerative competence were evaluated. Depending on cultivar, division rates of up to 40% were obtained in a KM-medium containing 0.5 mg/l of each BA, 2,4-D and NAA, and protoplast derived callus development occurred at high frequency. The apparent key step in this study was the application of the phenyl-urea herbicide thidiazuron (Mok et al. 1982) in a subsequent culture phase on solidified medium. In the German cultivar 'Mythos' shoot initials appeared with a frequency of 5-8% after 3 - 8 months (varying between individual calli), from which fertile plants could be recovered. A comparison of 30 different phytohormone compositions revealed that shoot morphogenesis occurred only in calli exposed to thidiazuron.

The extension of these studies to *Vicia narbonensis* (Tegeder et al. 1996) showed that also in this species plant regeneration from protoplast-derived calli via shoot morphogenesis can be achieved using the thidiazuron pathway. In addition, the protocol for the induction of somatic embryogenesis (originally developed for shoot tip-derived calli of *Vicia narbonensis*, Pickardt et al. 1989) was also successful. It is important to mention, that in *Vicia narbonensis* regeneration *via* somatic embryogenesis occurs with a considerable higher efficiency compared to shoot morphogenesis induced by thidiazuron. Unfortunately all attempts to induce somatic embryogenesis in *Vicia faba* were unsuccessful so far (Pickardt 1988 unpublished, Tegeder et al. 1995).

One of the problems in breeding of *Vicia faba* is its sexual incompatibility with other *Vicia* species, limiting broadening of the gene pool and the creation of new variability. Desirable agronomic traits like drought tolerance, insect and fungal resistance are available e.g. in *Vicia narbonensis* (Lawes et al. 1983). Hybridisation between *Vicia faba* and *Vicia narbonensis* by conventional techniques, including embryo rescue, always failed so far (Cubero 1982, Ramsay and Pickersgill 1986, Lazaridou and Roupakias 1993). The development of effective protocols for plant regeneration from protoplasts in both species establishes the prerequesites for attempting somatic hybridisation between *Vicia faba* and *Vicia narbonensis*. Moreover, the availability of transgenic lines containing different selection markers in both species (Pickardt et al.

1991, Böttinger et al. 2001, this study, see below) will allow an efficient selection of hybrid cells.

1.3 Transformation of grain legumes

Despite of the susceptibility of several grain legumes to the *Agrobacterium* infection, there are few grain legumes have been stably transformed by *Agrobacterium* transformation. This is due to the lack of sufficient regeneration protocols in many of the pulses. Grain legumes in general are considered as being more recalcitrant crops for *in vitro* manipulation than other species, particularly member of the *Solanaceae* (de Kathen and Jacobsen, 1995).

The most successful transformation protocols in grain legumes followed by recovering of transformed plants are those which using pre-excited meristematic cells either from embryonic axes like in pea (Schroeder et al. 1993; Bean et el. 1997), in *Cicer arietinum* (Fontana et al. 1993, Kar et al. 1996 , Kieseker 2000 and Krishnamurthy et al. 2000), *Lupinus angustifolius* (Molvig et al. 1997), from the shoot apical meristems like in *Lupinus angustifolius* (Pigeaire et al. 1997), *Lupinus luteus* (Li et al. 2000), using stem nodal segments or cotyledonary nodes in peas (de Kathen and Jacobsen, 1990; Nauerby et al. 1991, Davies et al. 1993). This transformation system is based on wounding the meristems prior to inoculation by *Agrobacterium* and inducing multiply axillary shoots by high concentrations of cytokinines. The advantage of these protocols are their simplicity and relatively short time required to produce the transgenic plants without a callus phase. This is also ensuring a low incidence of somaclonal variation.

The other transformation procedures are based on *de novo* regeneration by either organogenesis or somatic embryogenesis. Puonti-Kaerlas et al. (1990) achieved transgenic peas by organogenesis *via* callus formation using gene encoding hygromycin phosphotransferase as a selectable marker. Protoplast-derived mature plants of pea (Böhmer et al. 1995), and phynotypically abnormal transformants after polyethylenglycol (PEG)-mediated gene transfer (Böhmer 1995) were produced. Pickardt et al. (1991 and 1995) reported a transformation strategy based on somatic

embryogenesis *via* callus induction on etiolated shoot tips and epicotyl segments (see below).

There are efforts to improve the transformation of grain legumes through increasing the ability of *Agrobacterium* to penetrate the plant tissues by subjecting the explants to short periods of ultrasound in the presence of bacteria. This method called Sonication-Assisted *Agrobacterium*-mediated Transformation (SAAT: Tricker and Finer 1997) induces the formation of channels in the target tissues and facilitating bacterial access to the internal cells of the explants. This method has been applied to transform decapitated mature chickpea embryos and transformed shoots were able to regenerate *via* multiple shoots proliferation on TDZ medium (Kiesecker 2000). Transformation events of soybean embryogenic cell suspensions have been reported using this technique (Trick and Finer 1998).

There are also reports of direct DNA transfer into regenerable tissues of grain legumes. Christou et al. 1987 demonstrated that DNA-coated metal particles could deliver biologically-active DNA into organized soybean tissues, with subsequently recovery of stable transformants in the form of callus lines and transgenic roots. Afterwards McCabe et al. 1988 reported the recovery of the first genetically engineered soybean. Embryo axes have been used extensively as target tissues for direct transformation by particle bombardment in *Glycine max* (Sato et al. 1993; Christou 1990; Padgette et al. 1995), *Phaseolus vulgaris* (Russell et al. 1993; Aragao et al. 1996). Recently, embryonic cell suspension cultures of soybean were transformed with jellyfish *gfp* gene(Ponappa et al. 1999). The main limitation to this approach in some laboratories may be the limitation access to particle bombardment instruments (Christou, 1997).

1.4 *Vicia* spec. transformation:

The first transformation study with *Vicia faba* has been performed by Schiemann and Eisenreich (1989) by inoculation of the seedlings with *Agrobacterium rhizogenes* strains harbouring the binary vector pGSGluc1 transferring NPT II and GUS under control of the TR1/2 bi-directional promoter. GUS-positive roots were developed at

the inoculation sites. GUS activity has been monitored in the callus established from these roots. Shoot regeneration from these calli was not reported. In the same way a study has been done by Ramsay and Kumar (1990): cotyledons and stem explants from 8 different *Vicia faba* varieties were infected with *A. rhizogenes* harbouring the plasmid pBin 19 (Bevan et al.1984) transferring NPTII. Transgenic hairy root cultures have been established. The transgenic nature of established root clones were confirmed by hormone autotrophy and NPT dot blot assays. Attempts to initiate shoot morphogenesis in root cultures were not described. In 1993 Quandt et al. studied the gene expression in the transgenic root nodules of *V. hirsute*. They reported the induction of hairy roots on the wounded epicotyls of *V. hirsuta* by incubation with *A. rhizogenes.* Regeneration experiments were not attempted. Jelenic et al. (2000) inoculated stem segments of three broad bean cultivars with nine different *Agrobacterium* strains. With all strains tested only unorganized tumour tissue was obtained. Cultivars differed in their susceptibility to bacterium strains, and plant genotype vs. strain interaction was detected. Regeneration of transgenic shoots was not reported.

Pickardt et al. (1991) reported the recovery of five transgenic plantlets (T0) of *Vicia narbonensis*, transformed with the *Agrobacterium tumefaciens* strain C58C1/3850hpt carrying the gene for hygromycin phosphotransferase and the nopaline synthase gene. In this study the regeneration protocol for somatic embryogenesis (Pickardt et al. 1989) was successfully combined with the *Agrobacterium tumefaciens*-mediated gene transfer. The progeny analysis revealed a 3:1 segregation of the foreign genes, confirming the stable integration of the T-DNA at a single locus (Meixner et al. 1996). This protocol seems to be an efficient transformation system (Saalbach et al. 1994, Pickardt et al. 1995, Saalbach et al. 1995a/1995b, Pickardt et al. 1998, Weber et al. 1998, Czihal et al. 1999).

To date there is only one report on the successful *Vicia faba* transformation with the recovery of fertile transgenic plants by Böttinger et al. (2001). This transformation protocol is based on the *de novo* regeneration of shoot initials from dedifferentiated cells that was initially developed for plant regeneration from protoplasts using thidiazuron (Tegeder et al. 1995), combined with *Agrobacterium*-mediated transformation. Transgenic plants were recovered by inoculation of stem segments of

cultivar 'Mythos' with *Agrobacterium* EHA 101 or EHA 105, harbouring different binary vectors, followed by callus induction on MS-medium containing 0.5 mg/l of each thidiazuron, 2,4-D and NAA and 100 mg/l Kanamycin as selection 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 plants were recovered by micro-grafting. This process is still time consuming and needs 16-24 months to get seed producing primary transformants, relatively low efficiency and is prone to somaclonal variation.

1.5 Enhancing of the sulfur containing amino acids in grain legumes.

Grain legumes are an important protein source for food and feed. Dry seeds of *Vicia faba* contain at maximum 33 to 35% crude protein, Albumins represent 15% of total seed protein whereas Globulins contribute to the remaining 85%. The Globulins are composed of approximately 30% Vicilin and 70% Legumin. More than 50% of the methionine of field bean seeds proteins is contributed by albumins (Müntz et al. 1986).

Unfortunately such proteins are deficient in some essential amino acids e.g. methionine and cysteine for human and monogastric animal nutrition. This imbalance in amino acids composition restricts their biological value to 55 to 75% of that of animal protein (Müntz et al 1998). In many developing countries, like India and Egypt, the physical and mental development of children up to 4 years of age can be irreversibly retarded by the deficiency of essential amino acids in their diet (Müntz et al 1997). This deficiency in the sulfur containing amino acids made the grain legumes a target to improvement.

Traditional plant breeding was unable to solve this nutritional problem and could not significantly increase the essential amino acid content (Bliss 1990). So, novel breeding tools had to be developed which use molecular biology involving the modification of the genes, gene transfer and their expression in transgenic plants. This biotechnological approach provides a chance to solve this problem by manipulating individual genes without drastically decreasing protein yield (Wobus et

al. 1986). The data available support the hypothesis that insertion of one or few foreign genes do not adversely affect the agronomic characteristics of the host cultivars with the expression of the introduced gene. Thus breeding crop varieties by insertion of specific single or a few genes could be good a supplement to conventional crop improvement programs (Uchimiya et al. 1989).

There are two main strategies to improve the seed storage protein composition. The first approach to enhance the methionine and cysteine content of seeds is deregulating the biosynthetic pathway to increase the free amount of respective amino acids. Methionine and lysine are generated in a pathway starting from aspartate (Fig 1). The first step in this pathway is the phosphorylation of aspartate by the enzyme aspartate kinase (AK). Besides AK there is dihydrodipicolinate syntheses (DHDPS) playing a major role in the aspartate pathway of amino acid synthesis (Galili 1995; Brinch-Pedersen et al. 1996). Both enzymes are regulated by end product feed-back inhibition. Transformation of a gene coding for an isozyme insensitive to feed back end product inhibition could increase the content of free methionine, threonine and lysine significantly.

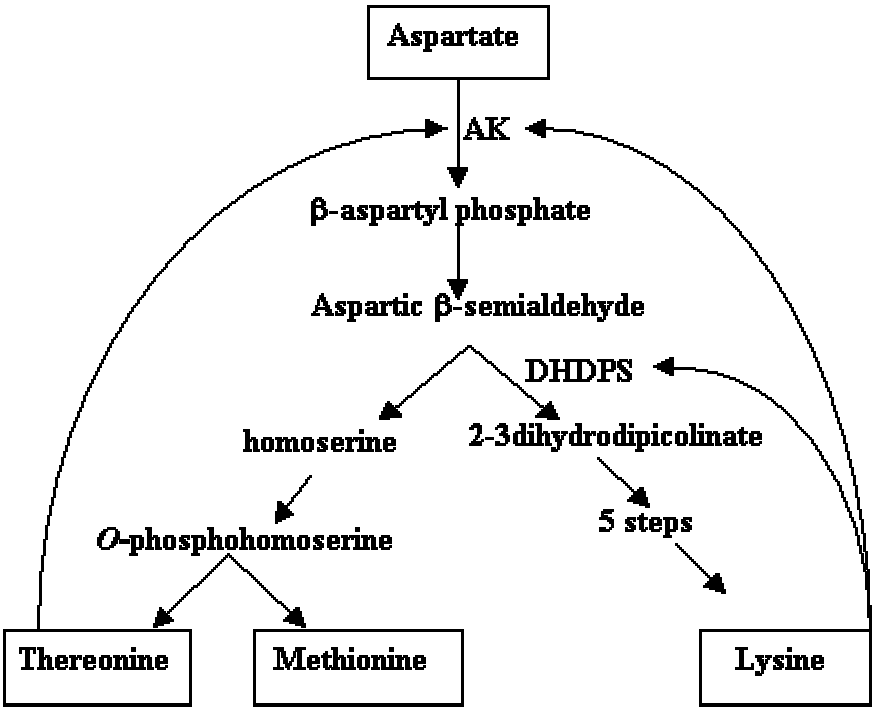

Fig (1): Biosynthetic pathway of the aspartate-family

Falco et al. 1995 reported that expression of insensitive bacterial DHDPS (*Corynebacterium dapA* gene) plus lysine-insensitive *E. coli* AK (*lysC* gene) in soybean transformants caused several hundred-fold increases in free lysine and increased total seed lysine content up to 5-fold. Also the free lysine and threonine were increased in tobacco transformants expressing the feed back insensitive AK and (or) DHDPS genes (Karchi et al 1993). The major disadvantage in applying this strategy to improve the amino acid composition as reported by Müntz et al 1998 is, that parallel to the accumulation of special free amino acids their degradation pathways are also activated.

The second strategy to improve the methionine content in the seed storage protein is the transferring of genes for methionine/cysteine rich foreign proteins. The ideal candidate genes for manipulation of methionine/ cysteine–rich protein genes in grain legumes are summarized in table (1).

Table (1): Methionine/Cysteine rich genes

Transgene source	Gene	Methionine (Met) %	Cysteine (Cys) %	Number of amino acids	References
Bertholletia excelsa (Brazil nut)	BNA	18	8	101	Sun et al. 1987
Helianthus annuus (sunflower)	*SFA8*	16	8	103	Kortt et al. 1991
Zea mays (corn)	10 kDa zein	22.5	3.9	129	Kirihara et al. 1988

Seed specific expression of a chimeric gene encoding a Brazil nut methionine-rich protein under phaseolin promoter in tobacco and canola transformants resulted in accumulation of the methionine-rich protein up to 8% in tobacco and up to 4% in canola of total seed proteins. The level of methionine in the seed proteins of transgenic plants has been significantly increased up to 30-33% (Altenbach et al. 1989, 1992.). Introduction of Brazil nut gene under the control of seed specific

promoter from *Arabidopsis* 2S albumin (AT2S1), lead to increases of the foreign protein to levels ranging between 1-2% of the total seed proteins of *Arabidopsis*, tobacco and *Brassica napus* (De Clercq et al. 1990). In *Arabidopsis thaliana* transformed with the brazil nut gene using a construct in which the transgene was present as a tandem duplication under the *At2S2* promotor (from the *Arabidopsis* 2S albumin) in the T-DNA, a 1.28% increase in the foreign protein was detected and this associated with 20% increasing in the methionine content (Conceicao et al. 1994).

Tu et al. 1998 tried to increase the methionine content of potato by transferring a cDNA encoding the methionine-rich protein of Brazil nut or its mutants encoding increased methionine contents *via* Agrobacterium transformation. The coding sequence was placed under regulation of CaMV 35S promoter and nopaline terminator. The expression of the brazil nut methionine-rich protein levels in the leaves among different constructs varied between 0.01% and 0.2% of total protein. The expression in the tubers was 2-4 fold lower than in the leaves.

On the other hand Guerche et al. (1990) found that the expression of Brazil nut 2S albumin gene (BNA) in the seed of transgenic *Brassica napus* was not enough to alter the amino acid composition. The methionine-rich protein was detected at the level ranging from 0.02 to 0.07% of total protein. The coding sequence of BNA was driven by a soybean lectin promoter.

Using this approach with grain legumes, Pickardt et al. 1995 transferred the BNA gene into *Vicia narbonensis* under the seed specific leguminB4 promoter from *Vicia faba* (Bäumlein et al. 1991). The expression of the BNA gene occurred in a seed specific manner and the foreign protein was presented at the level ranging from 1% to 4.8% of the total SDS-soluble seed protein. In further analysis for these transgenic plants, the methionine content of the salt-soluble protein fraction was determined in the transgenic seeds of T0 transformants (Saalbach et al. 1995a). It was found a threefold increasing in the methionine content in the transgenic seeds over the wild-type of *V. narbonensis*. In a previous report, Saalbach et al. 1994 described the expression of the synthetic BNA driven by CaMV 35S promoter (which permits gene expression in all organs) in transgenic *Vicia narbonensis*. The protein product was accumulated up to 0.1% of total soluble protein in leaves and 0.01% in seeds.

Transgenic expression of the BNA gene has been reported in the seed of soybean (*Glycine max*) by Jung et al -Pioneer Hi-bred International, Inc. USA 1997 (unpublished data). In that research the sulfur-rich 2S Brazil nut protein was accumulated to more than 10% of seed protein and resulted in nearly a 50% increase in the seed methionine content. Aragao et al. 1999 reported on the expression of the Brazil nut gene under control of CaMV 35S promoter in transgenic *Phaseolus vulgaris* that, the methionine content was significantly increased up to 23% of the seed salt soluble proteins of transgenic plants.

Due to the allergenicity of the 2S Brazil nut protein in some human aspects (Nordlee et al. 1996) however, the present research has shifted to using the sunflower 2S albumin gene (*SFA*8, Kortt et al. 1991) as a source for methionine rich protein. In an effort to study the possibility of using sunflower seed albumin (*SFA8*) in engineering a high methionine content in transgenic plants, Tabe et al. 1993 transformed tobacco plants either with *SFA8* gene or with a modified *SFA8* by addition of 3´end of the protein coding region of 18 nucleotides encoding the peptide; serine-glutamine-lysine-aspartate-glutamine-leucine (SEKDEL), this modified called $SFA8_{SEKDEL}$. Both types of the modifications were driven by the 35S promotor. This modification resulted in the accumulation of detectable levels of $SFA8_{SEKDEL}$ protein in the leaves of the transgenic plants, whereas there was no detectable SFA8 protein in the leaves of transgenic plants with *SFA8* gene without SEKDEL sequences. They hypothesized that addition of the SEKDEL peptide to the C-terminus of the SFA8 protein increases the stability of the protein by causing it to be retained in the endoplasmic reticulum of the leaf cells, thereby diverting it from its route to the vacuole.

Khan et al. 1996 studied the expression of a modified *SFA8* gene under the control of CaMV 35S in transgenic subterranean clover (*Trifolium subterraneum*). The *SFA8* gene was modified to contain a sequence encoding an endoplasmic reticulum-retention: threonine, serine, glutamine, lysine, aspartate, glutamine, leucine (TSEKDEL). It was found that the sunflower seed albumin (SSA) was accumulated in the leaves of T0 of transgenic plants at varying levels up to 0.3% of the total extractable protein. The accumulation level of SSA was increased with increasing the leaf age. In the same way Christiansen et al. 2000 introduced the coding sequence of the *SFA8* gene including an endoplasmic reticulum retention signal (KDEL) into

Trifolium repens. The *SFA8*$_{KDEL}$ gene was driven by different promoters, either the promoter of a gene encoding the small subunit of ribulose bisphosphate carboxylase (Rubisco) from *Arabidopsis thaliana* (A_{SSU}), the promoter of the gene encoding the small subunit of Rubisco of *Medicago sativa* (L_{SSU}) or CaMV 35S promoter. The highest level of sunflower seed albumin was 0.1% of total extractable leaf protein. Also the results showed that the promoter had substantial effects on the *SFA8* gene expression in the leaves with A_{SSU} > CaMV 35S > L_{SSU}. Transgenic tall fescue (*Festuca arundinacea* Schreb.) with a chimeric *SFA8*$_{KDEL}$ gene was obtained by Wang et al. 2001 in order to improve the protein quality of the forage grass for ruminant nutrition. The coding sequence of *SFA8*$_{KDEL}$ gene was constructed under the control of constitutive CaMV 35S promoter or light regulated wheat *Cab* promoters. The SSA was accumulated in transgenic plants up to 0.2% of the total soluble leaf protein.

To date the only successful report to enhance the sulfur containing amino acids in grain legumes was reported by Molvig et al. 1997. A seed specific expression of the *SFA8* in lupins (*Lupinus angustifolius*) under control of seed specific promoter from a pea *vicilin* gene was studied. The SSA was accumulated up to 5% of the total extractable seed protein and this associated with increasing the methionine level by 94% and unexpectedly reduction of cysteine level by 12%. In feeding trails with rats, the transgenic seeds of lupins gave significant increases in the live weight gain, true protein digestibility, biological value and net protein utilization, as compared with wild-type seeds. The same gene construct was transferred to chickpea and pea using *Agrobacterium*-mediated transformation and in all cases, the transgenic lines expressed detectable amount of SSA in the seeds of transgenic plants (Tabe et al. 1997 unpublished data).

During performing the current study two additional papers have been published which proved that the sunflower seed methionine-rich 2S albumin (SAA) is an IgE-binding protein (Kelly and Hefle 2000 and Kelly et al 2000).

1.6 Aim of the research

The aim of this research was to enhance the sulphur containing amino acids methionine and cysteine in the seed storage protein of *Vicia faba* by means of genetic transformation, thus improving their nutritional quality. Different transformation strategies either by direct gene transfer (particle bombardment) or by indirect transformation have been applied. In addition to *Agrobacterium*-mediated transformation (indirect gene transfer), two systems i.e. *de novo* regeneration protocol (Böttinger et al. 2001) and transformation of pre-existing meristems on embryo axes have been studied. Consequently, an efficient and reproducible *Vicia faba* transformation protocol based on the transformation of zygotic embryos transformation has been developed. Evaluation of the respective transformation protocols with different Elite *Vicia faba* cultivars (selected Egyptian and German cultivars) was also a part of this study.

The main strategy to engineer the seed storage protein composition is transferring a foreign gene encoding for a methionine rich protein. Sunflower 2S albumin *(SFA8)* gene (Kortt et al. 1991) is one of the best candidates to improve the nutritional quality of grain legumes. The coding sequence of the *SFA8* gene was driven by a seed specific promotor (*legumin*B promoter LeB4) from *Vicia faba* to promote seed specific expression of the transformed gene.

In a second approach, a multiple gene model was applied by transferring a gene coding for a methionine feedback insensitive form *LysC* gene from *E.coli* into faba genome and to co-transform the *SFA8* gene. Both genes are under control of seed specific promotors (phaseolin and legumin promoters, respectively). The study of the inheritance and expression of the introduced genes in the transformants progenies in the green house using different molecular and biochemical analysis completed this approach.

2.Materials and Methods

2.1 Source of chemicals and enzymes

1-Naphthaleneacetic acid (NAA)	(Sigma chemical/USA)
2,4-dichlorophenxy acetic acid (2,4-D)	(Sigma chemical/USA)
6-Benzylaminopurin (BAP)	(Sigma chemical/USA)
Agar–agar	(Roth/Germany)
Ammonium persulphate (APS)	(Serva/Germany)
B5-Medium	(Duchefa/Netherlands)
Bacto-Peptone	(Difco - Laboratories/USA)
Bacto-Tryptone	(Difco - Laboratories/USA)
Bacto-yeast	(Difco - Laboratories/USA)
Beef extract	(Difco - Laboratories/USA)
Bovine Serum Albumin BSA (fatty acid free)	(Sigma chemical/USA)
Combactam	(Pfizer/Germany)
CTAB (Hexadecyl Tri-Methylammonium)	(Merck/Germany)
DAB (diaminobenzidine tetrahydrochloride)	(Vector Laboratories/USA)
DIG-DNA labelling kit	(Boehringer/Germany)
DMSO (Dimethylsulfoxid)	(Sigma chemical/USA)
DNA markers	(Boehringer/Germany)
dNTPs	(Appligene)
Dye reagent concentrate (Bradford)	(Bio Rad/Germany)
EDTA	(Serva/Germany)
Ethidiumbromid	(Fluka/Switzerland)
Gelrite	(Roth/Germany)
H_2O_2	(Serva/Germany)
Kanamycin-Sulfate	(Sigma chemical/USA)
Kinetin	(Duchefa/Netherlands)
MS-Medium	(Duchefa/Netherlands)
Neeo-Agarose	(Roth/Germany)
Nylon membrane (positively charged membrane)	(Boehringer/Germany)
Phenol	(Roth/Germany)
Polyvinylpyroldon (PVP)	(Fluka/Buchs, Switzerland)

PPT	(Hoechst/Germany)
Primers	(MWG/Ebersberg)
PVDF membrane	(BioRad/USA)
Restriction endonucleases, restriction buffers	(Boehringer/Germany)
SDS	(Bio-Rad/USA)
Sucrose	(Merck/Germany)
Taq-polymerease	(Appligene)
Tetracycline	(Sigma/USA)
Thidiazuron (TDZ)	Riedel-de Haen
Ticarcillin	(Duchefa/Netherlands)
TMED	(Bio Rad/Germany)
Tris	(Serva/Germany)
Triton X-100	(Fluka/Switzerland)
VECTASTAIN® ABC Kit	(Vector Laboratories/USA)
X-Gluc.	(Duchefa/Netherlands)

2.2. Plant material

In the present study, 6 selected cultivars of *Vicia faba* i.e. Mythos, Albatross (obtained from Norddeutsche Pflanzenzucht/W.Lemke, Hohenlieth), Giza 2, Giza 429, Giza blanka and Giza 716 (obtained from Agricultural Research Center (ARE), Egypt) were used

Details of each cultivar are given in Table 1.

Table (1): Place of origin and special characteristics of *Vicia faba* cultivars

Cultivars	Place of origin	Type	Tolerance against parasitic weed	Tolerance against rust disease
Mythos	Germany	Minor	nd	nd
Albatross	Germany	*Minor*	nd	nd
Giza 2	Egypt	*Minor*	Susceptible	Susceptible
Giza 429	Egypt	*Minor*	Tolerant	Tolerant
Giza 716	Egypt	*Minor*	Tolerant	Tolerant
Giza blanke	Egypt	*Major*	Susceptible	Tolerant

Plant culture medium and culture condition

Standard MS medium (Murashige and Skoog 1962) and B5 (Gamborg et al. 1968) medium were used as plant culture medium, fortified with different combinations of growth regulators and antibiotics, depending on the transformation system that has been used and the respective culture stage. Media were solidified by 0.3% Gelrite and adjusted to pH 5.7 prior to autoclaving for 15 min at 121°C. Growth regulators were added to the media before autoclaving. To eliminate *Agrobacterium* growth after co-cultivation, 150mg/l Ticarcillin and 100 mg/l Combactam were used in the first stage of the transformation and for the next stages 100 mg/l Ticarcillin and 50 mg/l Combactam were added to the culture medium. The antibiotics were filter sterilized and added to the medium after autoclaving and cooling down to 50-60 °C. Petri dishes (Ø 9cm) and 250 ml-jars (8cm, covered with glass lids) were used as culture vessels. Petri dishes were generally sealed with parafilm. Cultures were kept at 21 °C under cool white fluorescent lights (80 $\mu mol.m^{-2}.s^{-1}$) with a 16h photoperiod.

Bacterial culture medium

Two types of bacterial culture medium have been used, YEB medium for *Agrobacterium*, and LB medium for either *E.coli* or *Agrobacterium*.

YEB-Medium

Beef extract	5 g/l
Yeast extract	1 g/l
Bacto – trypton	05 g/l
sucrose	5 g/l
Mg_4So_4 X 7 H_2O	0.495 g/l
pH	7.2

LB Medium

Bacto–trypton	10 g/l
Yeast extract	5 g/l
NaCl	10 g/l

2.3. Plasmids

Four plasmids, pGlsfa, pAN109, pTLsfa-GUS and pRT103gus were used for the transformation experiments.

- pGlsfa

This binary plasmid was constructed by taking the sunflower 2S-albumin (*SFA*8) gene as a *kpnI*/*sall* fragment from the pJsfa- plasmid (provided kindly by T.J.V. Higgins, CSIRO-Canberra-Australia) and subcloning it into the Lz7 plasmid for brining this gene under the seed specific LeguminB promotor (LeB4) from *Vicia faba*. A *Hind*III/*Xho*I fragment containing the cassette was then subcloned into the binary backbone Leg vic plasmid to create pGlsfa. This vector carried also the *bar* gene for a phosphinothricin acetyltransferase as a selectable marker controlled by the nopaline synthase *nos* promoter and g7 terminator (Fig 2) in its T-DNA. This plasmid

also carries a marker gene for the Kanamycin resistance serving for bacterial selection. The size of this vector is 15.102 kb. To do the transformation experiments the binary vector was transferred to *Agrobacterium* strain EHA 105.

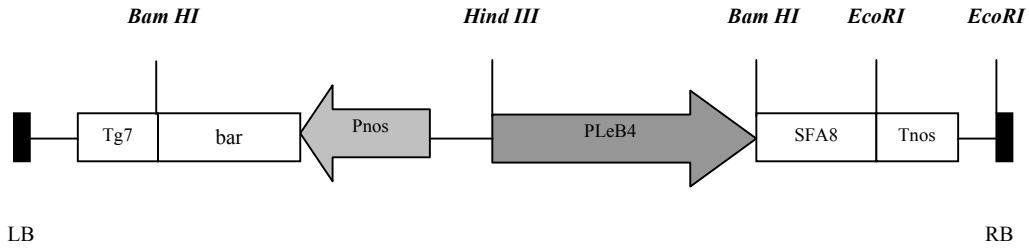

Fig (2): Map of the T-DNA regions of plasmid pGlsfa.

- pAN109

This binary plasmid has a size of 16 kb and was kindly provided by Gad Galili, (Weizman Institute/Rehovot, Israel), conferring P*nos-nptII* (as a selectable marker) and a mutated *lysC* gene from *E.coli* (coding for a feed-back desensitised aspartatekinase III, driven by the seed specific bean phaseolin promoter (Fig 3). Also, it is harbouring the coding region for tetracycline resistance which serves as a bacterial selection marker. It was transferred to *Agrobacterium* EHA 101 for the transformation experiments.

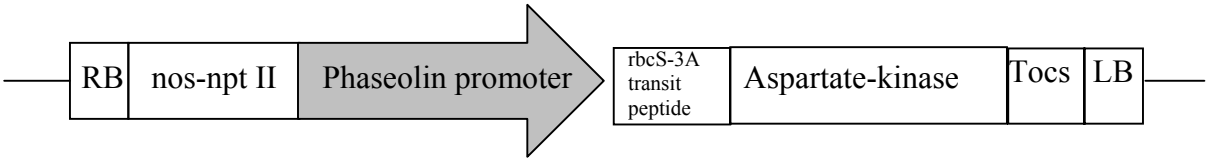

Fig (3): Map of the T-DNA regions of plasmid pAN109 (Karchi et al 1993)

-pRT103gus

The size of this plasmid is 5.1 kb. It contains ß-glucuronidase gene (GUS) under the control of the 35S promoter of the cauliflower mosaic virus and an ampicillin resistance gene. This plasmid was used for the direct transformation and transient expression experiments Fig (4)

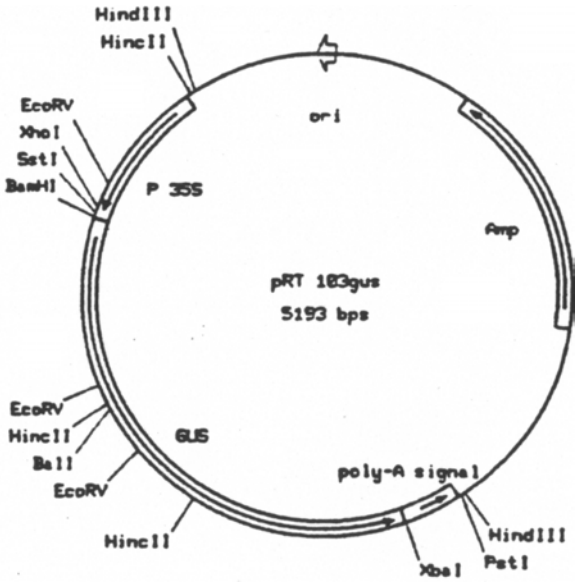

Fig (4). Structure of pRT103gus

- pTlsfa-gus

The size of this plasmid is 9.94 kb, it contains the sunflower 2S albumin gene *SFA8* under the LeB4 promoter, a ß-glucuronidase gene (GUS), as well as kanamycin and ampicillin resistance genes. This plasmid was used in direct transformation and transient expression assay (Fig 5).

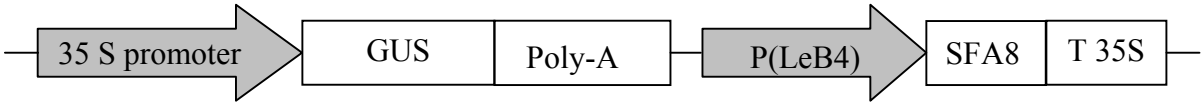

Fig (5). Diagram of the expression cassette of plasmid pTLsfagus

Plasmid transformation into *Agrobacterium*

1- Stored cells of competent *Agrobacterium* (500µl) were thawed on ice prior to transformation.
2- Competent *Agrobacteria* were mixed with 0.5 –1.0 µg plasmid DNA
3- The cells were incubated successively 5 min in ice, 5 min in liquid nitrogen and finally 5 min at 37°C
4- Add 1 ml YEB medium and incubate the bacterial culture 2-4 hr at 28°C under shaking (180 rpm)
5- Aliquots of 200 µl are plated on YEB–plates containing appropriate antibiotics.
6- After 2 days incubation, single colonies were picked and inoculated in liquid YEB medium for further analysis of the plasmid.

2.4.Transformation methods

In order to optimize and develop a reproducible transformation protocol, two transformation systems have been used namely direct (particle bombardment) and indirect (*Agrobacterium tumefaciens*) transformation. In comparison to *Agrobacterium*-mediated transformation, alternative two protocols have been studied: one protocol based on *de novo* regeneration using thidiazuron TDZ, (Böttinger et al. 2001), and as the second protocol one based on direct shoot organogensis from meristematic cells of mature and immature embryo axes (Schroeder et al. 1993).

In the direct transformation system, particle Gun/PDS 1000/He (Bio Rad) has been used to transfer the plasmid DNA to the mature embryo axes, cotyledonary nodes and cotyledons of cultivar ‘Albatross’.

2.4.1. Indirect transformation

2.4.1.1. *de novo* regeneration protocol

Source of the explants.

Seeds of faba cultivars (Mythos, Giza 2, Giza 429, Giza 719 and Giza blanka) were surface sterilized by immersion for 1 min. in 70 % ethanol and for 8 min in sodium hypochlorite solution (4% active chlorine) followed by washing 4-5 times with sterilized tap water, and soaked overnight in sterile tap water with shaking (90-95 rpm). Seeds were germinated in darkness on ½ MS-basal medium. Etiolated internode segments (lacking meristems) or leaf explants of the plantlets 10 days after germination were used for co-cultivation with A*grobacterium*. After cutting the primary shoot, secondary shoots arising from the cotyledonary node during the following weeks were used as explant sources as well.

Inoculation procedure and co-cultivation

Single colony of *Agrobacterium* strains were picked and grown overnight (16 hr) in liquid LB medium on shaker at 28°C and 180 rpm containing the appropriate antibiotics. Internodes of etiolated faba plantlets were immersed and cut into 2-3 mm-segments in a 1:5 dilution of bacterial suspension in liquid BNZ-medium (MS-medium, 3% sucrose, 0.5 mg/l of each BAP, NAA and 2,4-D, pH 5.7). The segments were briefly blotted on sterile filter paper and transferred to solid BNZ-medium for 2-3 days for co-cultivation in darkness at 20°C.

Selection and regeneration

Culture phase I:

Following co-cultivation, the explants were thoroughly washed in sterilised distilled water and placed on BNZ-Medium containing 150 mg/l Ticarcillin and 100 mg/l Combactam to eliminate *Agrobacterium* growth. After three to four days for recovering without selection pressure, the explants were subsequently transferred to

plastic Petri dishes containing BNZ-medium supplemented with 100 mg/l Ticarcillin and 50 mg/l Combactam and 2 mg/l phosphinothricin and (or) 100 mg/l Kanamycin (co-transformation experiments). The explants were sub-cultured every 2 weeks on fresh medium for a period of 3-4 months.

Culture phase II:

The resistant calli which were formed under selection pressure were transferred to MTN-medium (MS+3% sucrose, 7.5 mg/l thidiazuron, 0.75 mg/l NAA, 100 mg/l Ticarcillin and 50 mg/l Combactam) in 250ml jars. The calli were continuously subcultured on the same fresh medium every 3-4 weeks during a period of 6-12 months.

Culture phase III:

Shoot regeneration was seldom observed after about 8-12 months. Emerging shoots were transferred to elongation medium (MS-medium, 3% sucrose, 1mg/l BAP, 1mg/l GA3, 100 ml/l coconut milk, 100 mg/l Ticarcillin and 50 mg/l Combactam) in 250 ml jars.

Recovery of plants:

Healthy regenerated shoots were grafted onto 7-10 day old etiolated seedlings of untransformed (wild type) *Vicia faba* (grown under sterile condition as described in section “Source of the explants”).

Grafting was done according to Pickardt et al. 1995 by removing the epicotyl of the etiolated seedlings 1.5-2 cm above the cotyledonary node. The remaining part of the stem was split by a longitudinal cut ca.1 cm deep. Then, the transformed shoots were prepared as a graft using a very sharp razor blade in the shape of a wedge and inserted between the split epicotyl of the seedling (rootstock). The grafts were kept on ½ MS-medium in 250 ml glass container in light. Finally, any secondary shoots arose from the coyledonary node of the seedling during the following days were removed. After development of new leaves on the graft and the integration of the

graft and the rootstock, the grafted plants were transferred to the soil, acclimatized and placed in the greenhouse.

2.4.1.2. Faba embryo axes transformation system (direct shoot organogensis).

Plant material and transformation:

Source of the explants

Two types of explants (i.e. mature and immature embryo axes) were infected with the *Agrobacterium* strains. Immature pods of the *Vicia faba* cultivars were surface-sterilized with 70 % Ethanol for 3 min and subsequently washed with sterilized distilled water 4-5 times. Surface-sterilized mature seeds of the cultivar 'Mythos' were soaked overnight in sterilized tap water under gentle agitation. The embryo axes of both mature and immature seeds were wounded by removal of the root tips and slicing of the embryo axes to two or three segments longitudinally with a very sharp razor blade wetted by the *Agrobacterium* strain. Then the explants were incubated in the *Agrbacterium* suspension for 15-20 min (immature embryos) and 30 min (mature embryos).

Inoculation procedure

For preparation of the bacterial suspension culture, single colonies of the bacteria were grown overnight (16 hr) on a shaker at 28 °C and 180 rpm in LB liquid medium containing appropriate antibiotics. The *Agrobacterium* culture was diluted 1:5 with B5 liquid medium amended with 0.5 and 1 mg/l kinetin and 2,4-D, respectively. About 30-40 wetted explants were co-cultivated on solidified B5 medium with 0.5mg/l kinetin and 1mg/l 2,4-D at 25°C in dark for 3-4 days.

Recovery phase

After co-cultivation, the explants were subsequently washed thoroughly with sterile distilled water and placed on solidified MS (Murashige and Skoog 1962) 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 for 2 weeks without selection pressure.

Selection and regeneration phase

The explants were subsequently transferred to regeneration and selective 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 3-6 months. After at least 3-4 months of selection, the healthy resistant shoots were grafted onto etiolated seedlings wild type of *Vicia faba* to recover mature plants Fig 6.

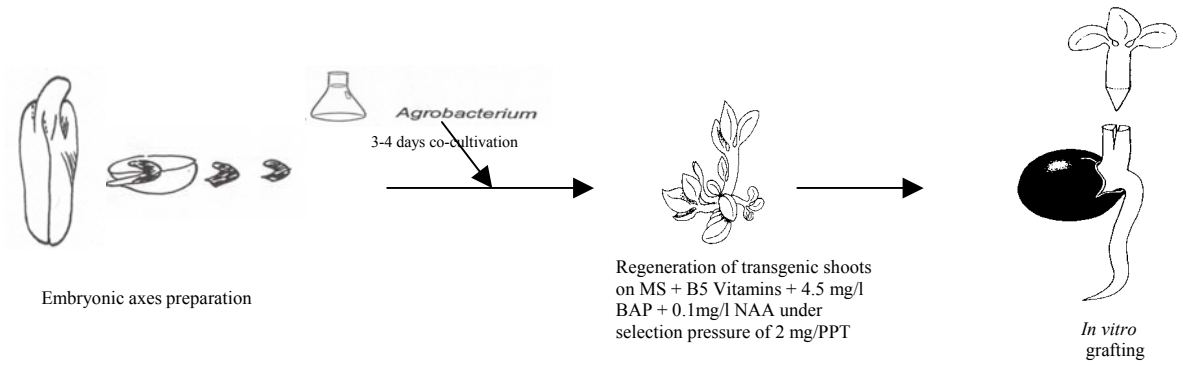

Fig. 6**:** *Agrobacterium* – mediated transformation of *Vicia faba* embryo axes

2.4.1.3 Co-transformation

An additional approach to increase the sulphur containing amino acid is to.

(a) transfer genes for methionine-rich foreign protein like *SFA8* gene from sunflower, and

(b) deregulate methionine synthesis.

In order to combine both strategies to improve the seed storage protein of faba bean by both strategies, co-transformation experiments have been done to introduce *SFA8* and *LysC* (encodes for feed back insensitive bacterial AK) genes together into faba genome.

The etiolated hypocotyls segments or the immature embryo axes explants of 3 cultivars of *Vicia faba* (Mythos, Giza 2 and Albatross) were co-transformed by a double *Agrobacterium* infection. EHA105/pGlsfa (Sunflower 2S Albumin and BASTA® resistance as a selectable marker) and EHA101/pAN109 (*lysC* / aspartate kinase under phaseolin promotor, Karchi et al. 1993 and *nptII* as selectable marker "kanamycin resistance"). Selection was done for only one of the markers (BASTA®) i.e 2 mg/l PPT with embryo axes transformation (see 2.3.1.2). After regeneration the selected plants were screened for the presence of the second marker. Also, selection was done with BASTA® or Kanamycin (100 mg/l) or by double selection (2mg/l PPT + 100mg/l Kanamycin) with the *de novo* regeneration protocol (Böttinger et al. 2001) see 2.4.1.1.

2.4.2. Direct gene transfer by particle bombardment

2.4.2.1 Plasmid DNA isolation from *E.coli*

For the direct transformation experiments, DNA of plasmids pTLsfa-gus and pRT103gus was isolated from *E.coli* as described below

1. Pre-culture of the *E.coli* strains (DH5α) which contains one of the mentioned plasmids was carried out in 5 ml of LB medium and 50 mg/l ampicillin for bacterial selection.
2. Cultures were grown overnight at 37°C and 200 rpm on rotary shaker.
3. Pre-cultures were inoculated in 300 ml LB medium+50 mg/l ampicillin and cultivated overnight at 37°C and 200 rpm on rotary shaker.
4. Plasmid isolation was performed after centrifugation of the bacterial culture for 15 min at 5000 xg, using NUCLEOBOND® protocol (Macherey-Nagel/ Germany) as described below:

    a) Carefully resuspend the pellet of bacterial cells in 12 ml of buffer S1 (50 mM Tris/HCl, 10 mM EDTA, 100 µg RNase A/ml, pH 8.0)
    b) Add 12 ml of buffer S2 (200 mM NaOH, 1% SDS) and mix the suspension gently by inverting the tube 6-8 times.

c) Add 12 ml of buffer S3 (2.80 M KAc, pH 5.1) and immediately mix the suspension gently by inverting the tube 6-8 times until a homogeneous suspension is formed. Incubate the suspension for 5 min on ice.
d) Centrifuge the suspension at high speed (>12.000 xg) at 4°C for 48 min. Directly after the centrifugation step carefully remove the supernatant from the white precipitate.
e) Equilibrate the NUCLEOBOND® cartridge AX 500 with 5 ml buffer N2 (100 mM Tris /H_3PO_4, 15% ethanol, 900 mM KCl, pH 6.3).
f) Load the clear lysate onto NUCLEOBOND® AX 500 cartridge equilibrated with buffer N2.
g) Wash the cartridge 2X with 12 ml of buffer N3 (100 mM Tris/H_3PO_4, 15% ethanol, 1150 mM KCl, pH 6.3).
h) Elute the plasmid DNA with 12 ml of buffer N5 (100 mM Tris/H_3PO_4, 15% ethanol, 1000 mM KCl, pH 8.5).
i) Precipitate the purified plasmid DNA with 0.7-0.8 volume of isopropanol, preequilibrated to room temperature. Centrifuge immediately for 30 min at high speed (>15.000 x g) at 4 °C. Briefly dry the DNA pellet (about 5 min at room temperature).
j) Redissolve the Plasmid DNA in TE-buffer (1mM Na_2EDTA, 10mM tris/HCl,pH 8) at a final concentration of 1 µg/µl.

2.4.2.2 Explants transformation of the cultivar 'Albatross'

Embryo axes, cotyledonary nodes and cotyledons were obtained from mature seeds of the cultivar 'Albatross'. Explants were prepared as described in 2.2.1.2 and pre-cultured on MS medium supplemented with 5 mg/l BA and 0.1 mg/l NAA in the centre of plastic Petri dishes overnight in darkness. To coat the gold particles (1.1µm) with DNA, the calcium–spermidine method has been used (Klein et al. 1987).

Preparation of the gold particle and coating.

1. Vortex 60 mg gold particles (1.1 µm) for 5 min in 96% Ethanol.
2. 1 min centrifugation, remove the supernatant and repeat two times.
3. Resuspend the particles in 1 ml sterile bidistilled water and vortex for 5 min.
4. 1 min centrifugation, remove the supernatant.
5. Resuspend the particles in 1 ml bidistilled water, repeat twice.
6. Aliquot 100 µl into 1.5 ml tubes while vortexing the suspension.
7. Add10 µl Plasmid-DNA into 100µl Gold particle suspension (60 mg /ml), 100 µl 2.5M $CaCl_2$, 40 µl 0.1M spermidin-free base and mix by slow vortexing in an Eppendorf tube for 5 min.
8. Let the particles settle down for 10 min and remove the supernatant.
9. Wash the pellet with 100% ethanol by slow vortexing for 10 min.
10. Let the particle settle down (10 min), and remove the supernatant, resuspend the pellet in 100 µl 100% ethanol (slow vortexing).
11. Pipet 6-7 µl of the particle suspension in the middle of the macrocarrier while vortexing the suspension continuously.
12. Air drying of the macrocarrier under low humidity.

Particle bombardement device and bombardement conditions

The standard bombardment procedure followed the PDS-1000/He manufacturer's instruction (BioRad) with minor modifications. This system is based on the acceleration of DNA/particles directly in helium pressure flow. The helium pressure utilized was 1800 and 2000 psi. The distance between the macrocarrier/DNA and the target explants (Target distance) varied from 3 to 9 cm. The number of bombardments per Petri dish were 1, 2 or 3 times.

After bombardment the Petri dishes were incubated for 3-4 days in darkness, afterwards the dishes were incubated under 16 h photoperiod. Every 3-4 weeks the explants were passed to fresh medium in glass jars (250 ml).

The regenerated shoots were subjected to GUS analyses by taking 4-6 mm sections from the basal end of the shoots (after about 2-3 months from bombardment) and analysed for gus-expression with X-Gluc as described by Jefferson (1987).

2.5 Regeneration and transformation frequencies

-Regeneration frequency is defined as the ratio between the number of regenerated shoots and the initial number of calli.

-Transformation frequency is defined as the ratio between the number of stable independent transformants and the initial number of explants.

2.6. Analysis of the transformants

2.6.1 β-glucuronidase assay (GUS assay)

Transient GUS gene expression was determined a few days after transformation of the explants. 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc) is the best substrate for histochemicheal localization of the β-glucuronidase activity in the tissues and cells (Jefferson 1987). This substrate produces a blue precipitate at the site of enzyme activity. The histochemical staining was performed according to Jefferson (1987). Transformation efficiency was expressed as the number of blue spots per bombarded explant. The transformed explants were incubated overnight at 37°C in the substrate buffer which contains 0.5 mg X-Gluc/ml. After staining, the plant explants were soaked in 70% ethanol to allow the detection of the blue stain.

0.1 M Phosphate buffer (pH 7.4)

3.1 g $NaH_2PO_4(x1H_2O)$
13.7g $Na_2HPO_4(x2H_2O)$
in 1 liter H_2O

Substrate buffer

40 mM Phosphate buffer (pH 7.4)
10 mM Na-EDTA
0.1% Triton X-100
0.5 mM K_3 [Fe $(CN)_6$]
0.5 mM K_4 [Fe $(CN)_6$]

2.6.2 PAT Enzyme Assay

A modified enzyme assay was carried out for the detection of phosphinotricin acetyltransferase (PAT) activity, encoded by the *bar* gene (isolated from *Streptomyces hygroscopicus* (Thompson et al. 1987). Expression of the *bar* gene confers resistance to phophinothricin (PPT), the active molecule in the herbicide BASTA® by acetylation of PPT and producing an inactive product, N-acetyl-PPT.

Procedure:

1- Homogenize of 20-50 mg leaf explants or callus tissues in an 1.5 ml Eppendorf tube with 40-100 µl ice cold extraction buffer.
2- After 15 min centrifugation at 4°C and 14000 rpm, add 15 µl from the supernatant to 2 µg of phosphinotricin and 2 µl of ^{14}C-Acetyl-CoA (60mCi/mmol).
3- Incubate the reaction for 60 min at 37 °C
4- Spin down the tubes for 2 min at 14 000 rpm
5- Apply 4µl of the supernatant onto a marked position of a TLC plate.
6- Run the Chromatography for 2-3 hr. in a chromatography tank containing chromatography buffer (1-propanol and NH_4OH (25 % NH_3) in a 3:2(v/v) ratio).
7- Air dry the plate for 60 min
8- Expose the TLC-plate to an X-ray film (Kodak X-OMAT LS) in a light-safe cassette for 24-48 hr at room temperature.
9- Autoradiograph the film to record the signal

PAT extraction buffer

50 mM Tris-HCl, pH 7.5
2 mM Na-EDTA
300 mg/l Dithiotreitol (DTT)
300 mg/l BSA
150 mg/l Phenylmethylsulfonylfluorid (PMSF)

2.6.3 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 200g/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 were tested by the same method or young plants were sprayed (around 3-4 weeks after germination) by the same BASTA® solution. Resistance of leaflets or plants were scored after 7-10 days.

2.6.4 Southern blot analysis

DNA was isolated from young leaves tissue of faba bean plants using 'Plant DNeasy mini kit' (Qiagene). 25 μg genomic DNA from transgenic plants were digested by the following restriction endonuclease (Boehringer Mannheim/Germany):

***Hind III*:** This enzyme has only one restriction site in the T-DNA of the pGlsfa plasmid, and was used to determine the copy number of the integrated genes in the plant genome.

***Bam HI*:** which has two restriction sites in the T-DNA of the plasmid pGlsfa. By this enzyme it is possible to obtain the full length of the legumin promotor (LeB4) and the *bar* gene under the *nos* promoter with a length of 3.5 kb.

Restriction reactions were done according the manufacturer's instructions, with overnight incubation at 37°C. The digested genomic DNA was electrophoresed through 0.8% (w/v) agarose gel in a running buffer of TAE to separate the DNA fragments according to the size.

2.6.4.1 Southern blotting (transfer)

Separated DNA fragments cannot be conserved in the agarose gel. Therefore the DNA has to be transferred to a nylon membrane (positively charged membrane-Boehringer/Germany). The transfer was performed by means of neutral capillary transfer according to Sambrook et al (1989) or by vacuum blotting (alkaline transfer) using a Vacuum Gene XL- Apparatus (Pharmacia) according to the manufacturer's instruction.

Capillary transfer under neutral conditions

1. Prior to blotting, the DNA in the gel was depurinated in order to guaranty a complete transfer of large fragments of DNA and this has been performed by soaking the gel for 5-7 min on 0.25 M HCl with gentle agitation.
2. DNA denaturation was done by soaking the gel in alkaline buffer (1.5 M NaCl, 0.5 N NaOH) for 30 min, in order to have single strands of the DNA to allow hybridisation.
3. Neutralization of the gel through neutralization buffer (1.5 M NaCl and 1 M Tris/HCl pH 7.5) for 2 times, each 15 min.
4. Finally The DNA was blotted by capillary transfer method using 20X SSC (3 M NaCl, 0.3 M sodium citrate, pH 7.0) as a transfer buffer overnight.
5. DNA was fixed to the membrane by backing for 30 min at 120 °C.

2.6.4.2 DNA DIG labelling

A 1100 bp *bar* coding region was isolated by *HindIII* / *BamHI* double digest of pGPTV Gloc. plasmid or a 750 bp *SFA8* coding region was isolated by *SalI* /*BamHI* double digest of pTLsfa-gus plasmid. The digested plasmids were separated according to the size through 0.8% TBE agarose gel. Subsequently, the *bar* or the *SFA8* fragments were isolated from the gel using the QIAquick Gel Extraction Kit Protocol according to the manufacturer's instructions (Qiagene).
The isolated fragments were DIG labelled using DIG high prime labelling kit (Boehringer Mannheim/Germany) as described in the following protocol.

1- Dilute of 1 µg DNA template in H_2O for a total volume of 16 µl
2- Denature the DNA template for 10 min in boiling water, and quickly chill it on ice.
3- Add 4 µl DIG-High prime, mix and briefly centrifuge.
4- Incubate the reaction tube at 37°C over night.
5- Incubate the reaction for 10 min at 65°C to terminate the reaction.

Subsequently, the yield of DIG-labelled template was estimated through spot tests with a DIG–labelled control by comparing serial dilutions of the DIG-labelled sample and the different known concentrations of the DIG-labelled control (Boehringer Mannheim/Germany) which were spotted side by side on a nylon membrane. Subsequently, the membrane was colorimetrically detected following the manufacturer's instructions

Procedure:

1. Make a predilution of the DIG-labelled control DNA by mixing 5 µl DIG-labelled control DNA with 20 µl DNA dilution buffer (10 mM Tris-HCl, pH 8.0, 50 µg/ml DNA from herring sperm), the final concentration is 1ng/µl.
2. Make serial dilutions (100 pg/µl, 10 pg/µl, 1 pg/µl, 0.1pg/µl and 0.01pg/µl) of the (prediluted) control.
3. Make serial dilutions of the experimental probe
4. Spot 1 µl of the diluted controls on a nylon membrane.

5. In the second row, spot 1 µl of the corresponding dilutions of the experimental probe.
6. Fix the nucleic acids to the membrane by baking for 30 min at +120°C
7. wash the membrane briefly in washing buffer (100 mM maleic acid, 150 mM NaCl; pH 7.5; 0.3% (v/v) Tween® 20)
8. Incubate the membrane in blocking solution (1% (w/v) blocking reagent, dissolved in washing buffer) for 30 min at room temperature.
9. Dilute the Anti-DIG-alkaline phosphatase 1:5000 in blocking solution.
10. Incubate the membrane in the diluted antibody solution for 30 min at room temperature.
11. wash the membrane 2x, 15 min per wash in washing buffer at room temperature.
12. Incubate the membrane in detection buffer (100 mM Tris-HCl, 100 mM NaCl; pH 9.5).for 2 min.
13. Mix the 45 µl NBT solution and 35 µl BCIP solution in 10 ml of detection buffer (colour substrate solution).
14. Pour off the detection buffer and add the colour substrate solution. Allow the colour development to occur in the dark (few minutes).
15. When the spots appear in sufficient intensity, stop the reaction by washing the membrane with sterile H_2O for 5 min.
16. Compare spot intensities of the control and the experimental dilutions to estimate the concentration of the experimental probe.

2.6.4.3 Prehybridisation and hybridisation

The membrane was prepared for probe hybridisation by prehybridisation with pre-hybridisation solution (5X SSC, 0.1% sodium-lauroylsarcosine, 0.02% SDS, 5% blocking reagent and 50% formamide) for 3-4 hr at 42°C under gentle agitation. which blocks non specific nucleic acid-binding sites. Afterwards, the pre-hybridisation solution was discarded and the membrane was hybridised with hybridisation solution having the same composition as the prehybridisation solution but the DIG labelled probe at a concentration of (50 ng/ml) added, which was previously denatured by heating at 100°C for 10 min and quickly cooled down in an ice bath.

The membrane was hybridised overnight at 42°C in a hybridisation oven. Finally, the membrane was washed two times by washing buffer 1 (2X SSC and 0.1 % SDS) for 15 min at room temperature with gentle shaking, followed by the second wash step (2 times 15 min at 65°C) under gentle shaking in washing buffer 2 (0.5X SSC and 0.1% SDS).

2.6.4.4 Detection

The chemiluminescent detection of the hybridised probe encompassed three steps. The first step is blocking the membrane with blocking solution for at least 1 hr at room temperature with gently agitation to prevent non specific attraction of antibody to the membrane

The second step is incubation of the membrane with a dilution of anti-Digoxigenin (1:10000) in blocking solution for 30 min at room temperature with gentle rotary shaking.

As the third step, the membrane carrying the hybridised probe + bound antibody was incubated with a chemiluminescent substrate (CSPD® Boehringer Mannheim/Germany) for 5 min. This substrate produces a light signal on the site of the hybridised probe

2.6.4.5 Autoradiography

The membrane was exposed between 3 hr – 24 hr at room temperature to Kodak X-OMAT LS autoradiograph film to record the chemiluminescent signal.

2.6.5 PCR analysis

DNA was isolated for PCR analyses according the method of Sul and Korban (1996) from young leaves using cetyldimthylethyl-ammonium bromide (CTAB) with minor modifications.

CTAB-extraction buffer

2% CTAB
10mM EDTA
50mM tris/HCl (pH 8.0)
1% PVP
4M LiCl

DNA isolation protocol

1- Frozen plant material (100-300mg) was ground under liquid nitrogen and homogenized with one volume (300-400 µl) of extraction buffer.
2- The homogenate was incubated for 10-15 min at 65°C, during the incubation time the homogenate was mixed 2-3 times by inverting the tube.
3- Afterwards one volume (700 µl) of chloroform-isoamylalcohol solution (24:1) was added and carefully mixed.
4- Short centrifugation (4 min) at maximum speed (14000 rpm) in a bench-top centrifuge.
5- The supernatant was again extracted with chloroform/isoamylalcohol mixture and centrifuged.
6- The supernatant was transferred to fresh 1.5 ml-tubes.
7- The chlorform/isoamylalcohol extraction was repeated until clarification of the supernatant, usually after the second time.
8- Subsequently, the DNA was precipitated by about 3 volumes of ice cold absolute ethanol, and carefully mixed 2-3 times.
9- 10 min centrifugation and the pellets were washed with 70% ethanol to remove any salts. Finally the DNA was resuspended in H_2O.

The presence of sunflower 2S albumin (*SFA8*) or *lysC* genes was assayed using Polymerase Chain Reaction (PCR).

PCR reaction mix

0.5 µg	Genomic DNA
1.5 mM	$MgCl_2$
2.0 units	Taq polymerase (Appligene)
30 pM	each primer
80 µM	dNTP (each)

The reaction was done in total volume of 100 µl

PCR programme

94 °C	1 min
57°C	1 min
74°C	2 min

the reactions were run for 30 cycles

Primers

The *SFA8* sequence (750 bp) was amplified using the following primers

SAF: 5'-ACT AGT ATG GCA AGG TTT TCG ATC-3'
SAR: 5'-GAG CTC TTA CAT TTG GCA TGG TTG-3'

The following primers were used to amplify the coding sequence (1056 bp) of *lysC*.

Primer 1: 5'-GAT TTT GAC GCC ATG AAC CGC A-3'
Primer 2: 5'-TGT CAG CAA CGT ATC GCC AGT GG-3'

2.6.6 RNA isolation and reverse transcriptase (RT)-PCR analysis:

The transformed clones were analysed by RT-PCR to characterize the expression of *SFA8* and legumin genes at the RNA level. Total RNA was isolated from frozen (–70 °C) immature seeds (30 days after pollination) either by RNeasy mini kit (QIAGEN) or by the following protocol.

Total RNA isolation from immature seeds of Vicia faba

1- The frozen embryos (-70°C) were ground under liquid nitrogen using RNase free mortar and pestle in an ice bath.
2- The plant materials were extracted and well homogenised with one volume (700µl) of extraction buffer and one volume (700µl) Phenol/Chloroform/ Isoamylalcohol PCI (25:24.1).
3- The homogenate was transferred to RNase free 2ml tubes and mixed by vortexing, followed by 2 min centrifugation at 0°C and 14000 rpm.
4- The aqueous phase was transferred to new tubes and extracted again with 700 µl PCI and centrifuged for 5 min.
5- Again, the aqueous phase was transferred to a new tube and the RNA was precipitated by 1/20 volume of 3M Na-acetate and 0.8 volume ice cold isopropanol at 0°C for 30 min.
6- Centrifugation for 10 min at 0°C and 14000 rpm in a bench-top centrifuge.
7- The supernatant was discarded and the pellet was washed with ice cold 70% ethanol.
8- RNA was resuspended in one volume of RNase free H_2O in ice bath. Afterwards, one volume of 4 M LiCl was added and the mixture was incubated at 4°C overnight for total cellular RNA precipitation.
9- RNA was precipitated by 30 min centrifugation at 4°C and 14000 rpm.
10- The pellet was washed with 1 ml 2 M LiCl, followed by centrifugation and washing the pellet by 70% ethanol.
11- Finally, RNA was resuspended in RNase free H_2O (treated with DEPC and autoclaving).

RNA extraction buffer:

1M Tris (pH 7.5)
1% SDS
1 mM Na-EDTA
10mM ß-Mercaptoethanol

All the buffers and solutions were treated with 0.1% DEPC except Tris-solutions. Afterwards, the buffers were autoclaved for 20 min at 121°C. Mortars and pestles were treated by baking in oven for 5 hr at 160 °C. This is to inactivate the RNases.

c-DNA construction:

The first strand cDNA was constructed by Expand Reverse Transcriptase kit according to the manufacturer's instruction (Roche/ Germany) using either specific reverse primers for sunflower 2S albumin (SAR) or for leguminB4 (Leq2) as an internal control or using oligo$(dT)_{18}$ primers. The cDNA was amplified using the same PCR protocol and primers for *SFA8* amplifications to detect the transcript as described before. The following oligonucleotides were used for RT-PCR amplification of the legumin transcript (1200 bp):

LeguminB4 primers

Leq1: 5´-TCC AGA GCT CCA CAG TCA CAA TGT CCA AAC-´3
Leq2: 5´- TGC ACA GCT GTT GCA CTC CTT AGC ATG ATC-´3.

2.6.7 Western blot analysis

For western blot analysis, proteins were extracted from either mature or immature seeds (30 days after pollination) of clones transformed with the pGlsfa plasmid or co-transformed with pAN109 plasmid.

Total protein extraction protocol:

1. Homogenisation of 20 mg seed flour or 200 mg immature cotyledons in 200 µl extraction buffer.
2. 10 min incubation in boiling water bath (*SFA8* transformants) or at 65°C for *lysC* transformed plants.
3. Centrifugation for 10 min at 4°C and 14000 rpm.
4. The supernatants were transferred to fresh 1.5 µl tubes. Protein concentrations were determined according to Bradford (1976).

Protein extraction buffer (*SFA8* transformed plants)

25mM TRIS-HCl (pH 9.0)
0.5% SDS
10% Glycerol

In order to separate the protein according to the molecular weight, 40-80 µg protein were mixed with loading buffer (1:1) and loaded on a 15% SDS polyacrylamide gel (Laemmli 1970) for *SFA8* transformed plants or into 10% SDS polyacrylamide gel for AK transformed plants

Protein extraction buffer (AK transformed plants)

56 mM Na_2CO_3
2% SDS
12% Sucrose
2 mM Na_2EDTA
50 mM DDT

Loading buffer

0.25M Tris (pH 6.8)
6% SDS
20% Glycerin
0.2M DTT
0.05% BPB

Western blotting

After running the gel at 20mA for about 60 min, it was soaked for 15 min in transfer buffer with agitation. The polypeptides separated in the SDS gel were blotted onto PVDF membrane (BioRad) using the mini trans blot Apparatus (BioRad) for 60 min at 100V according to the manufacturer's instructions. Prior to blotting the membrane was treated for 15 sec in methanol followed by 2 min in water, finally the membrane was rinsed for 5 min in transfer buffer.

Transfer buffer

20 mM Tris
150 mM Glycine
20% Methanol

Immunodetection of the transgenically expressed proteins

Detecting the antigen in the immunoblot is achieved by binding a specific antibody to the proteins which were immobilized on the membrane as described in the following protocol

1- After blotting, the membrane was rinsed in TTBS buffer for 30 min with gentle shaking.
2- The membrane was probed with either an anti-sunflower 2S albumin-specific polyclonal antibody or with an anti-aspartate kinase-specific polyclonal antibody overnight with gentle agitation.
3- The membrane was washed 3x 10 min with TTBS to remove all unbound antibody.
4- The blot was incubated with the labelled (biotinylated) secondary antibody (Anti rabbit IgG), diluted up to 1:2000 in TTBS for 60 min with agitation at room temperature.
5- The membrane was washed 3x each 10 min with TTBS to remove unbound antibody.

6- Transfer the blot to a dilute solution of VECTASTAIN® ABC Vector Laboratories/ USA (one drop of reagent A and B to 20 ml TTBS). Incubate the blot in this dilution for 2 hr with gentle agitation.
7- Transfer the membrane to the chromogenic substrate for peroxidase to develop the blot and visualize the specific bands.
8- Finally wash the blot with 2 changes of distilled water for 10 min and allow the membrane to air dry.

TTBS buffer

0.1 M Tris (pH 7.4)
0.1% Tween 20
0.155 M NaCl

Chromogenic substrate

0.1 M	Tris–HCl (pH 7.4)
0.4 mg/ml	$NiCl_2$
0.009%	H_2O_2
0.01%	DAB

2.6.8 Amino acid analysis of seed proteins from transgenic faba bean

The amino acid composition of transgenic faba seed meal was determined by an oxidation based method. The seed coat was removed and the seeds were ground using mortar and pestle. For the analysis at least 1000mg from seed meal of each transgenic clone (2-3 seeds) were sent to Degussa-Hüls AG/Germany. Three clones were analysed by this way i.e T2 and T3 of clone Mfka/1, clone ME1/2/1 (T3) and clone MRE2/3/14 (T2).

Transgenic clones were designed as described below:

ME1/2/1 M: is the Mythos cultivar, E1 experiment code, 2: Clone number 1: Graft number.
Mfka/1 M: Mythos cultivar, fk: Co-transformation experiment, a: Experiment code, 1: Graft number
MfkE1/2/2 M: Mythos cultivar, fk: Co-transformation experiment, E1: Experiment code, 2: Clone number and graft number
MRE2/3/12 M: Mythos cultivar, R: Regenerated plant from mature embryo axes, E2: Experiment code 3: clone number, 12: Graft number
M/7/85 M: Mythos cultivar, 7: Experiment number, 85: clone Number
M/7/39 M: Mythos cultivar, 7: experiment number, 39: clone number
M/1/339 M: Mythos cultivar, 1: experiment number, 339: clone number
Mfk2/3 M: Mythos cultivar, fk: Co-transformation experiment, 2: Experiment number, 3: clone number
Mfk2/13 M: Mythos cultivar, fk: Co-transformation experiment, 2: Experiment number, 13: clone number
Mfk1/35 M: Mythos cultivar, fk: Co- transformation experiment, 1: Experiment number, 35: clone number
G2/1/2 G2: Giza 2 cultivar, 1: experiment number, 2: Clone number
G2/1/23 G2: Giza 2 cultivar, 1: experiment number, 23: clone number
AB1/2/3 A: Albatross cutivar, B1: Experiment code, 2: clone number, 3: Graft number
Abk1/5/1 A: Albatross cultivar, bk: Co-transformation experiment, 1: Experiment number, 5: clone number, 1: Graft number
Abk3/4/3 A: Albatross cultivar, bk: Co-transformation experiment, 3: Experiment number, 4: Clone number, 3: Graft number

3.Results

3.1 Transformation experiments

3.1.1 Indirect transformation

3.1.1.1 *de novo* regeneration protocol

The *Agrobacterium* transformation experiments under a *de novo* regeneration based protocol, were done with five faba bean cultivars (Mythos, Giza 2, Giza 429, Giza 716 and Giza blanka). *Agrobacterium tumefaciens* strain EHA 105 harbouring the pGlsfa plasmid with the *SFA8* gene driven by the seed specific legumin promoter and a *bar* gene as a selectable marker was used. The control experiments showed that PPT at 2 mg/l totally suppressed callus development from wild type faba explants (epicotyl segments and leaf explants) cultured on MS-medium supplemented with 0.5 mg/l of BAP, NAA and 2,4-D. All the explants died. In a series of 10 transformation experiments, two types of the explants were inoculated with *Agrobacterium*, i.e. leaves and epicotyl segments. A total of 7580 epicotyl and 552 leaf explants from 5 cultivars were co-cultivated with the *Agrobacteria*. Whitish resistant callus started to proliferate after about 1-2 months on the surface of a number of explants (Fig 7A). Within 3 to 4 months after culturing the explants on selective medium, 4.3-31.59% of the explants produced resistant calli. PPT resistant calli with a diameter about 5-10 mm were transferred to the regeneration medium (phase 2) without selection pressure to increase the callus viability to regenerate (Fig 7B). Shoot regeneration occurred after 6-12 months on MS medium supplemented with 7.5 mg/l TDZ, 0.75 mg/l NAA (Fig 7C). *Agrobacteria* were controlled by media supplementation with 100 mg/l Ticarcillin and 50 mg/l Combactam. 15 shoot primordia of 3 cultivars were recovered from different calli clones within this period. Table (2) summarizes the callus induction and shoot regeneration frequencies from all the cultivars and explants tested. During the callus induction period (phase I), All the cultivars tested produced PPT resistant callus from both types of explants (stem segments and leaf explants).

Table (2): Callus induction and shoots regeneration from *Agrobacterium*–infected explants

cultivar	type of explants	total number of the explants	number of resist. calli	mean[a] (%)	maximum[a] (%)	minimum[a] (%)	regeneration frequency (%)
Mythos	stem	2486	270	9.35±8.13	24.3	2.3	10 (3.7)
	leaf	166	54	31.59±13.25	45.0	14.3	0
Giza 2	stem	1684	100	5.94±5.80	16.7	1.7	2 (2.0)
	leaf	153	29	20.17±11.44	37.5	9.1	0
Giza 429	stem	2022	109	4.81±3.52	9.4	0.5	1 (0.9)
	leaf	134	28	20.67±17.82	53,6	0.0	0
Giza blanka	stem	679	63	10.57±5.62	15.6	4.5	0
	leaf	59	16	22.43±21.27	42.3	0.0	0
Giza 716	stem	709	31	4.3	5.3	3.3	1(3.2)
	leaf	40	15	37.1	40.9	33.3	1(6.6)

[a] The mean (± standard deviation), maximum and minimum of the callus induction under selection pressure (number of callus clones obtained per total number of explants treated) were calculated.

Out of the 15 shoot primordia regenerating clones, only 8 shoot clones of cultivars 'Mythos, Giza 2 and Giza 716' produced shoots with suitable size for grafting. On the other hand, cultivar 'Giza blanka' didn't express any ability to regenerate neither from stem segments derived callus nor callus derived from leaf explants.

Shoot primordia were transferred to 1 mg/l BAP medium (phase 3) where they began to elongate and further shoot development took place (Fig 7-D). Because of the very low rooting percentage of the regenerates, the shoots which reached a suitable size (within 2-4 months on phase 3) were carefully grafted onto non-transgenic root stocks of *Vicia faba* as described in 2.4.1.1 to recover a whole plant (Fig 7-E).

Afterwards, the plants were transferred to soil for acclimatization and later transferred to the greenhouse. Eight clones of 3 cultivars were transferred to the greenhouse for further plant development and production of T1 seeds. Only 4 clones produced normal flowers and pods with seeds. The time needed to obtain T1 seeds by this process is about 16–24 months.

Some regenerated clones showed morphological abnormalities, such as dwarfing and the formation of abnormal flowers and subsequently abnormal (or no) pods (Fig 8A-D). Some plants showed narrow leaves and very weak stems with weak apical dominance (Fig 8B). In another case callus regenerated into etiolated shoots lacking chlorophyll and no further development (Fig 8C). Early (*in vitro*) flowering was also observed, in this case the flowers failed to set seed *in vitro*, and if these plants were transferred to the greenhouse, they did not produce normal pods.

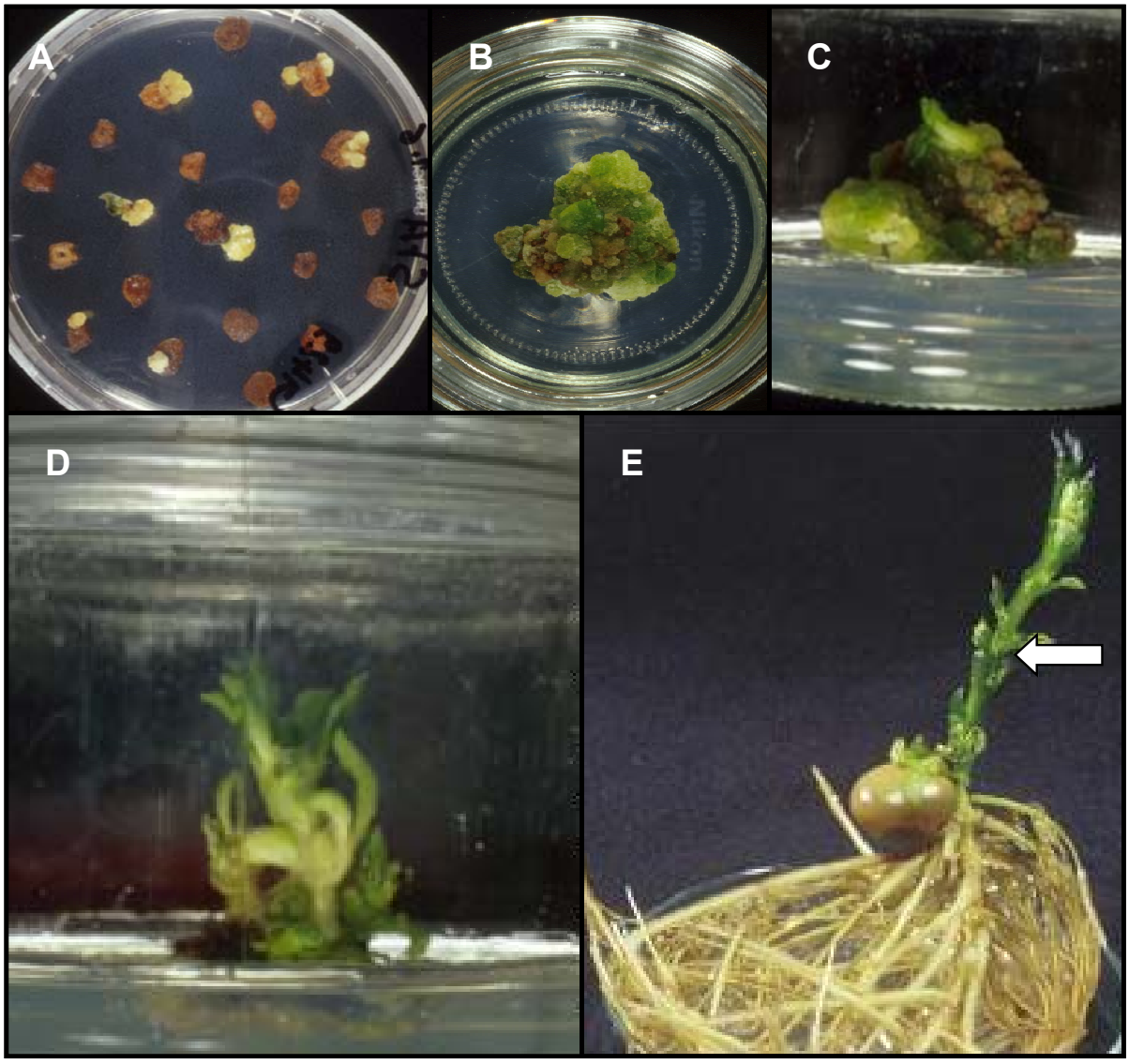

Fig (7A-E): Transformation of faba bean epicotyls and leaves explants. A) Initiation of resistant callus under selection pressure (Phase I)**. B)** Callus proliferation on TDZ medium (Phase II). **C)** Shoot regeneration (Phase II). **D)** Shoot elongation on BAP medium (Phase III). **E)** Recovering of a whole plant by *in vitro* micro-grafting (arrow)

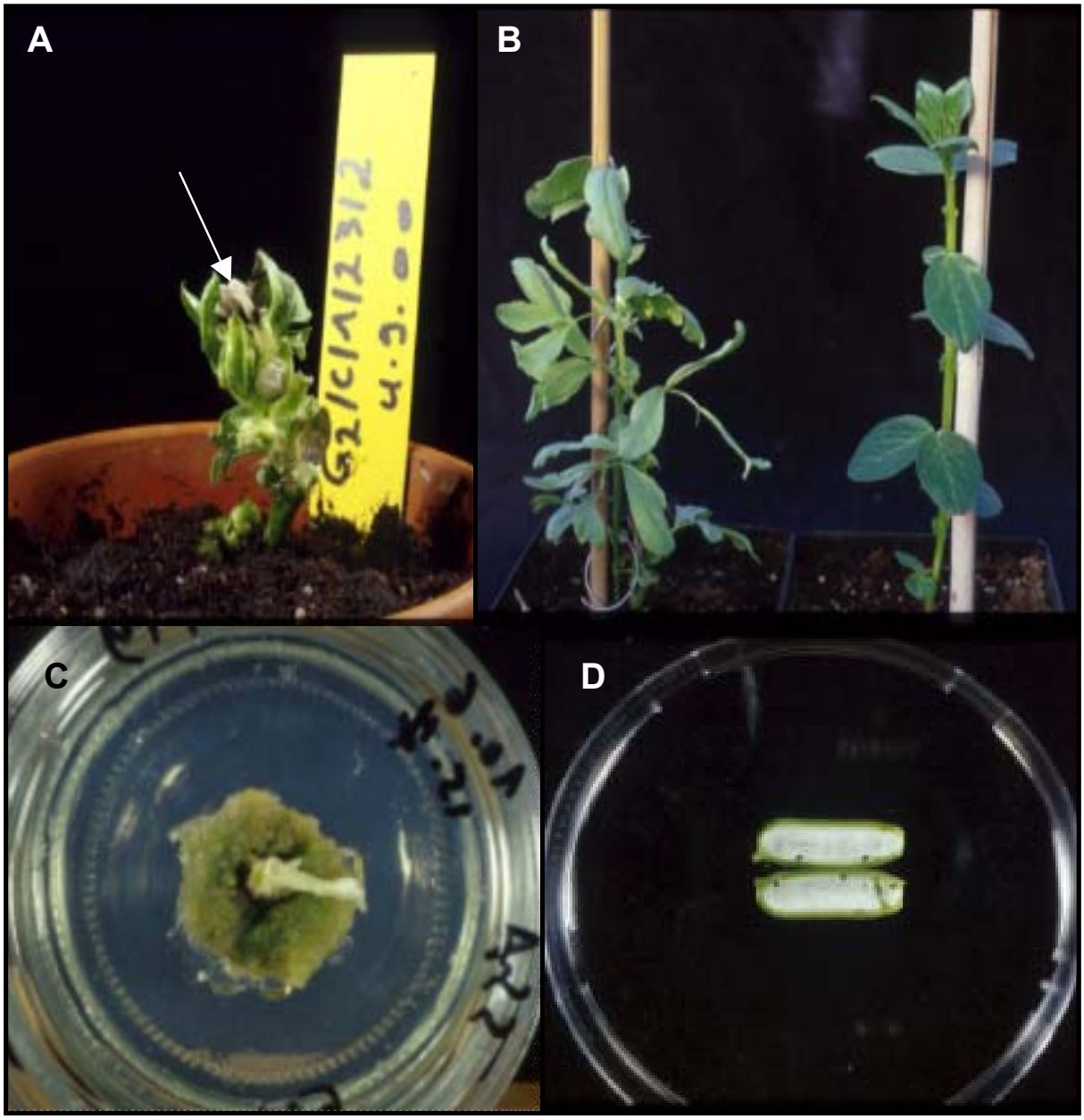

Fig (8 A-D): Morphological abnormalities of some primary transformed clones. **A)** dwarfness and abnormal flowers (arrow). **B)** regenerated plant (left) with abnormal leaves in comparison with normal plant (right). **C)** regeneration of etiolated shoot. **D)** abnormal pod lacking seeds.

3.1.1.2 Faba bean embryo axis transformation (direct shoot organogensis).

The feasibility of the transformation strategy developed in this experiment was initially evaluated by monitoring the number of regenerated shoots from the embryo axis explants cultured on high BAP concentration medium (4.5 mg/l). It was worked out, that around 4-5 shoots regenerated from each explant.

In a 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* (coding for a feed-back desensitised aspartatekinase III under the phaseolin promoter and *npt* II-kanamycin resistance as a selectable marker). A total of 1967 embryo axis explants from total 6 cultivars were co-cultivated with the *Agrobacterium* strains (Fig 9A). 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 other plasmid.

After 3-4 weeks of culturing the embryonic axes on selective medium, all control explants were dead. On the other hand, the transformed explants started to form callus and to regenerate (*via* organogenesis) and about 3-4 shoots appeared from each explant (Fig 9B) on MS salts medium supplemented with B5 vitamins, 4.5 mg/l BAP, 0.1 mg/NAA, 100 mg/l ticarcillin, 50 mg/l combactam and 2 mg/l PPT. The shoots selected were grafted under *in vitro* condition and finally transferred to the greenhouse to set seeds (T1), Fig 9C-E.

Data presented in table (3) summarize the results derived from 15 independent transformation experiments. A total of 7 stable independent transformants (*SFA8*) of 2 cultivars (Mythos and Albatross) have been recovered. Regarding the co-transformation experiments, transformed 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 (3) percentage of transformation in different *Vicia faba* cultivars

Cultivar	Explants type	Plasmids	Number of co-cultivated explants	Number of putative transformed lines (*SFA8*)	Number of transformed lines (*SFA8*)	Transformation frequency (*SFA8*) (%)
Mythos	Immature embryo axes	pGlsfa	141	8	1	0.71
		pAN 109 and pGlsfa	100	7	2	2.00
	Mature embryo axes	pGlsfa	220	11	1	0.45
Albatross	Immature embryo axes	pGlsfa	667	11	1	0.15
		pAN 109 and pGlsfa	380	03	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

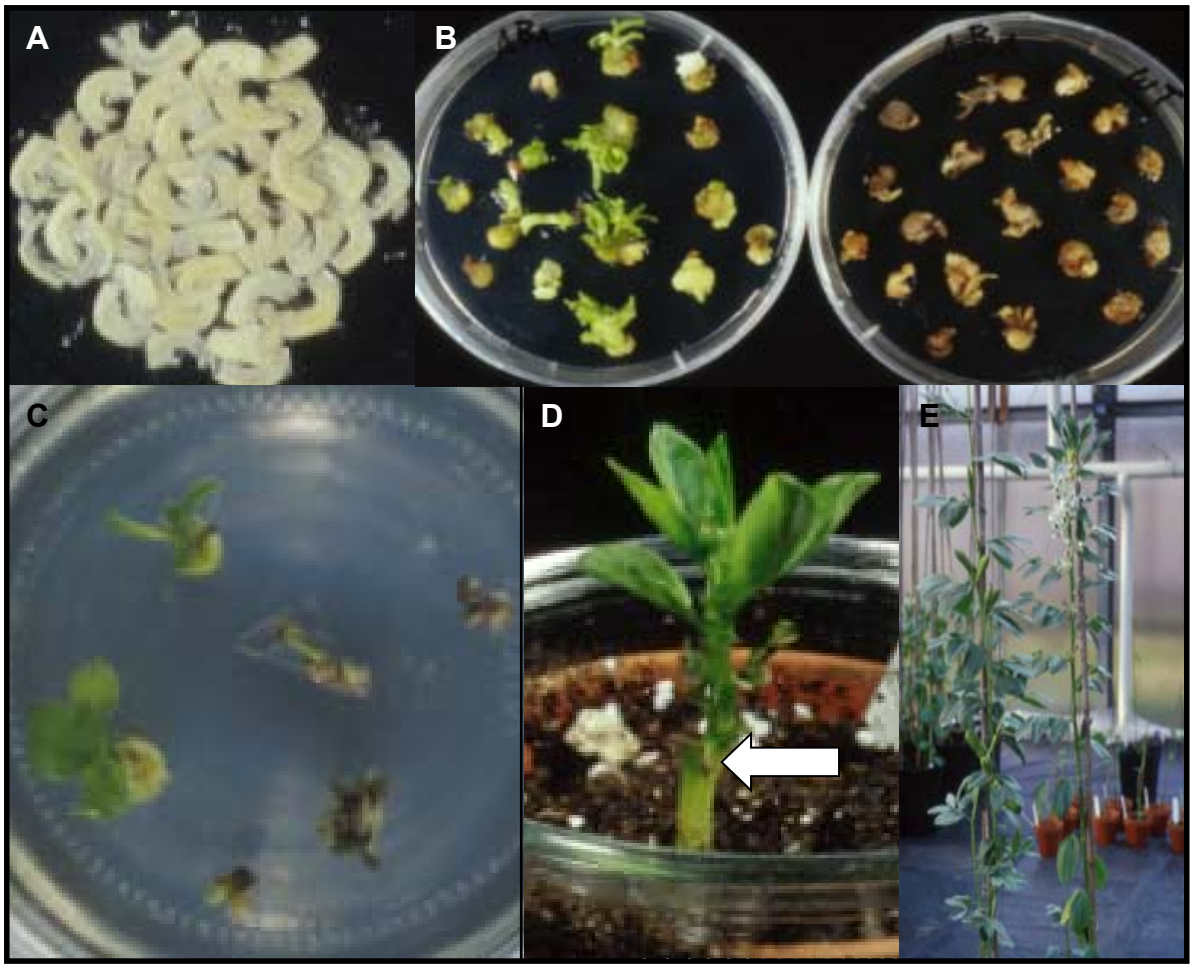

Fig (9 A-E): Regeneration of transgenic faba bean plants. **A)** Explant segments derived from embryonic axes. **B)** Multiple shoot regeneration under selection pressure (left), all the control explants (WT) were dead (right). **C)** Further selection between PPT resistant and susceptible regenerated shoots on medium containing 2 mg/l PPT. **D)** Grafted shoot transferred to the soil. **E)** Transgenic plants with flowers and pods.

3.1.2. Direct transformation using particle bombardment

The objective of this experiment is to analyse the possibility of using direct gene transfer based with faba bean explants and to monitor transgene expression in the transformed explants. Initial experiments were conducted using 3 different faba tissues (i.e. cotyledons, cotyledonary nodes and embryonic axes) from one genotype (Albatross). The efficiency of plasmid DNA uptake has been estimated by analyzing transient expression of the GUS gene in the cells of treated explants. Because a high transient GUS expression was observed in all tissues tested (Fig 10 A-C) and to simplify the results, the date presented below from one type of the explants i.e. embryo axes.

The effect of the bombardment numbers per Petri dish (0.7 µg/plsmid DNA per bombardment) and the distance between the macro-carrier (DNA) and the explants (target distance) on the GUS expression was determined with the plasmid pRT103gus (table 4). It was found that the number of cells per embryo with visual GUS activity in all the tested parameters were high.

Data presented in table 5 show the results of 5 independent transformation experiments. The results exhibit that higher acceleration pressure (2000 psi) reduced the number of regenerated shoots. This is due to the increasing of bombardment shock, tissue injury and subsequently cell death.

The GUS activity was determined after 2 months in the regenerated shoots from explants transformed with pTlsfa-gus. The number of shoots showing GUS expression was extremely low and the transformed cells per shoot which expressed the GUS activity were very low as well (Fig 10 D).

Table (4): Transient expression of the GUS gene in embryonic axes after bombardment.

Target distance (cm)	no. of bombardments per Petri dish (each containing 20 explants)	no. of explants	mean number of GUS expression unit per explant
9	2	60	43
6	2	130	58
6	3	40	67
3	2	40	53
3	1	50	30

Table (5): Efficiency of faba transformation via particle bombardment

acceleration pressure (PSI)	no. of bombardments per Petri dish (each containing 30 explants)	no. of explants	survival rate (%)	shoots per explant after 4 weeks	no. of shoots subjected to X-Gluc staining (after 2 months)	stable GUS positive plants
1800	2	549	90.0	3.4	285	none
1800	3	339	94.1	4	179	none
2000	3	250	90.8	2.5	132	none

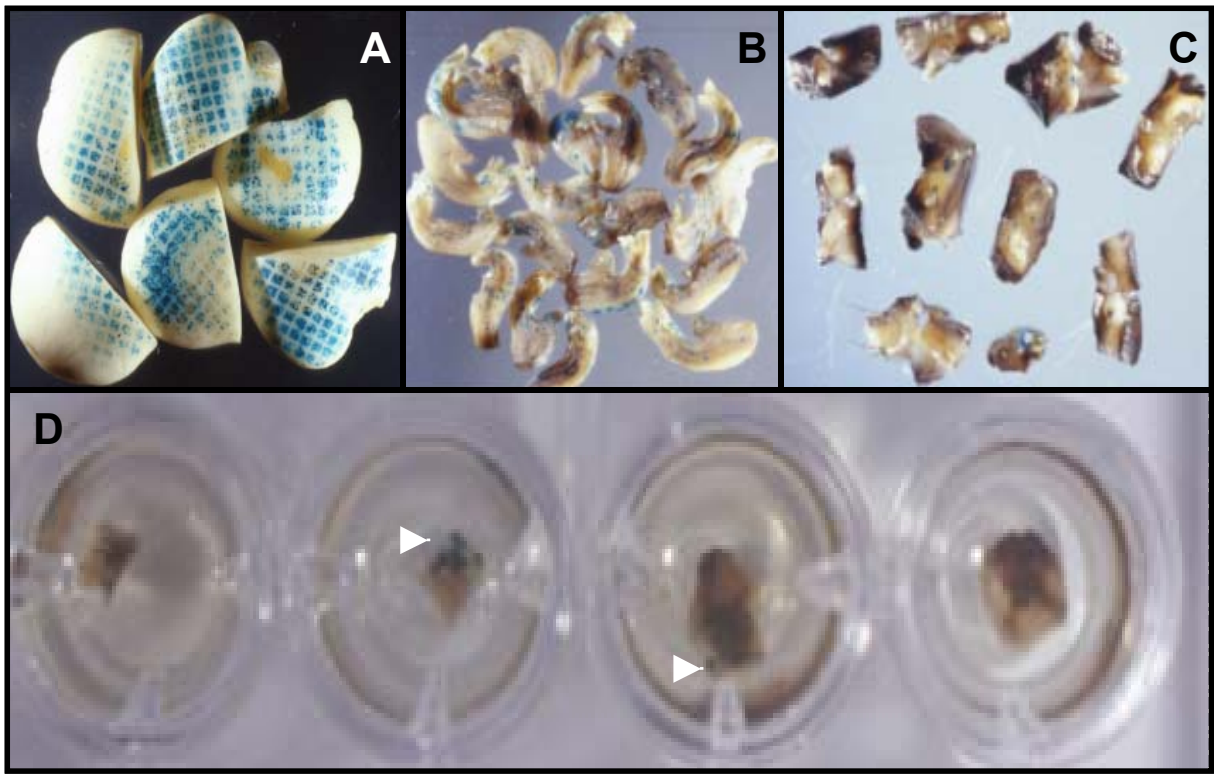

Fig (10 A-D): GUS expression in different faba bean tissues after bombardment. **A-C)** Transient expression **(A)** in cotyledons explants, (B) in embryonic axes, **(C)** in codyledonary nodes. **D)** GUS expression in putative transgenic shoots (arrows) 2 months after bombardment.

3.1.3 Co-transformation

The co-transformation experiments using EHA105/pGlsfa (*SFA8* and *bar* genes) and EHA101/pAN109 (*lysC* and *nptII* genes) were done as descried in 2.4.1.3. This was performed to determine the possibility of co-introducing *SFA8* and *lysC* genes coming from two independent binary vectors in one transgenic plant. This is in order to study the possibility of combining the strategies of enhancing the sulfur containing amino acids. Transferring of a gene coding for methionine-rich foreign protein (*SFA8*) and simultaneous engineering of the seeds amino acid metabolism (*lysC*) in a double transformant.

Resistant calli were proliferated under different selection regimes, either PPT or kanamycin or both in combination (see 2.4.1.3). Data presented in table (6) summarize the results of 3 independent co-transformation experiments carried out with the cultivars 'Mythos and Giza 2'. From the 2255 co-transformed explants (stem segments and leaf explants), 215 selected calli clones were obtained from both cultivars. 7 shoots were regenerated from one genotype (Mythos). Out of 46 selected calli clones on 2 mg/l PPT (the selectable marker in pGlsfa plasmid), 2 resistant clones were regenerated. On 100 mg/l kanamycin selection (the selectable marker in pAN109) 4 resistant shoots out of 89 selected callus clones were detected. From 31 selected calli clones on double selection only one regenerated clone was obtained. The regenerated shoots were micro-grafted to recover the whole plants. Subsequently these plants have been subjected to biochemical, molecular and genomic analysis.

Table (6): Co-Transformation with pGlsfa and pAN109 by a double *Agrobacterium* infection

Cultivars	Type of selection	Treated explants	Selected calli (%)	Regeneration frequency
Mythos	PPT	435	46 (10.6)	2(4.3)
	Kanamycin	470	89 (18.9)	4(4.5)
	PPT + Kanamycin	420	31 (7.3)	1(3.2)
Giza 2	PPT	400	21 (5.3)	0
	Kanamycin	470	26 (6.1)	0
	PPT + Kanamycin	60	02 (3.3)	0

3.2 Analysis of the transformants

3.2.1 Selected calli clones

3.2.1.1 Phosphinotricin Acetyl Transferase Assay (PAT Assay)

The *bar* gene encodes for the enzyme phosphinotricin acetyl transferase, which inactivates the herbicide phosphinotricin (BASTA®) by acetylation (De Block et al. 1987; Murakami et al. 1986; Thomposon et al. 1987). The expression of the *bar* gene in the calli clones selected on media containing PPT was initially tested by the PAT-assay (2.6.2). Four PPT resistant callus clones were randomly chosen and tested for the activity of the enzyme phosphinotricin acetyl transferase. Three clones expressed PAT activity. The level of the expression is varying in independent clones (Fig 11). In clone M/5/16 no PAT activity could be detected.

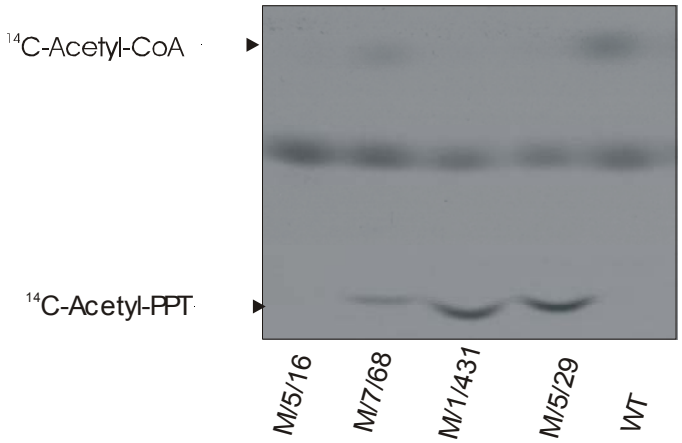

Fig (11): PAT activity in PPT selected calli clones and wild type (WT)

3.2.2 Primary (putative) transformants (T0)

3.2.2.1 Analysis of the marker gene expression

PAT Assay

Expression of the selectable marker (*bar* gene) was confirmed by the presence of PAT enzyme activity in the leaf materials of primary transformants (T0). The PAT activity was detected in many clones of cv 'Mythos, Albatross, Giza 2 and Giza 716'. The intensity of the expression was varied from high to very low expression (Fig 12). Only 10 clones of 3 cultivars (i.e. Mythos, Albatross and Giza 2) showed a high PAT expression. Some cultivars such as Giza 429 and Giza blanka didn't express any activity for PAT enzyme and these are probable negative.

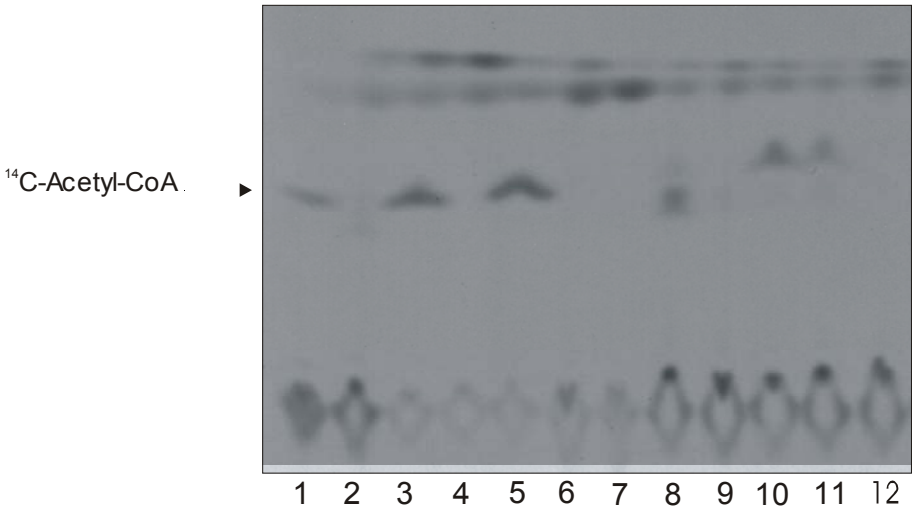

Fig (12): PAT activity in leaf extracts of faba primary transformants (T0). Lines 2 and 12 are from the negative control (extracts from untransformed faba bean WT). Lines 1,3 to 11 are leaf extracts from different primary transgenic faba of 2 cultivars (Giza 2 and Mythos).

Leaf paint

The primary transformants were analysed by testing the expression of the *bar* gene in the greenhouse by applying the leaf painting assay. Leaflets of non-transformed and transformed plants were tested for tolerance to the herbicide (BASTA®) at a concentration of 300-400 mg/l ammonium glufosinate. In addition, the whole plants were tested for BASTA® tolerance by spraying the whole 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 (Fig 13A-B), in contrast to the treated non-transformed plants showed leaflets which were completely necrotic.

A  B 

Fig (13 A-B): Herbicide (BASTA®) resistant transgenic faba bean. A) Leaf painting test showing the resistance of transgenic leaf to BASTA® application. The right-hand leaflet of each pair was marked and untreated. The left-hand leaflet of each pair was painted with 1:500 dilution of BASTA®. The left-hand leaflet pair was from a transgenic plant, and the right-hand leaflet pair was from a non-transgenic plant. **B)** Resistance of transgenic plants to spraying with a dilution 1:500 of BASTA®, non-transgenic plant (right) died after spraying, while the transgenic plant (left) continued the normal growth.

3.2.2.2 Integration of the introduced genes into the *Vicia faba* genome

Genomic analysis of 3 selected independent primary transformants (T0) from different transformation experiments, which showed a weak PAT activity, i.e. G2/E1/1/1, G2/E1/2/1 and G716/E1/1/1 (designed as genotype/experiment number/clone number/graft number) which regenerated from embryo axes and did not show resistance in the leaves painting test with BASTA®. This was done in order to confirm the integration of the T-DNA and to examine the copy number of the integrated T-DNA. Genomic DNA was digested with *HindIII* which cuts the pGlsfa vector only once or without digestion (with clone M/7/85, which expressed high PAT enzyme activity), transferred to nylon membranes, and hybridised with the DIG labelled *bar* gene (Fig 14 and 15). The hybridisation profile confirmed the integration of the T-DNA into the genome of the putative transgenic plants. Each clone exhibits a different integration pattern with different copy number varying from 2 to 1 copies. (clones G2/E1/1/1 and G2/E1/2/1, respectively). Because of the enormous genome size of *Vicia faba* (13.3 pg/1C), hybridisation signals of single genes are very faint and hard to detect (e.g. G2/E1/2/1 and G716/E1/1/1).

In respect to the undigested DNA blot, the hybridisation signal which was observed confirm that more than one copy of the T-DNA had been integrated (band intensity in comparison with the positive control) into the genome of clone M/7/85/1 (Fig 15) and with *BamHI*-digested genomic DNA from the same clone (Fig 21).

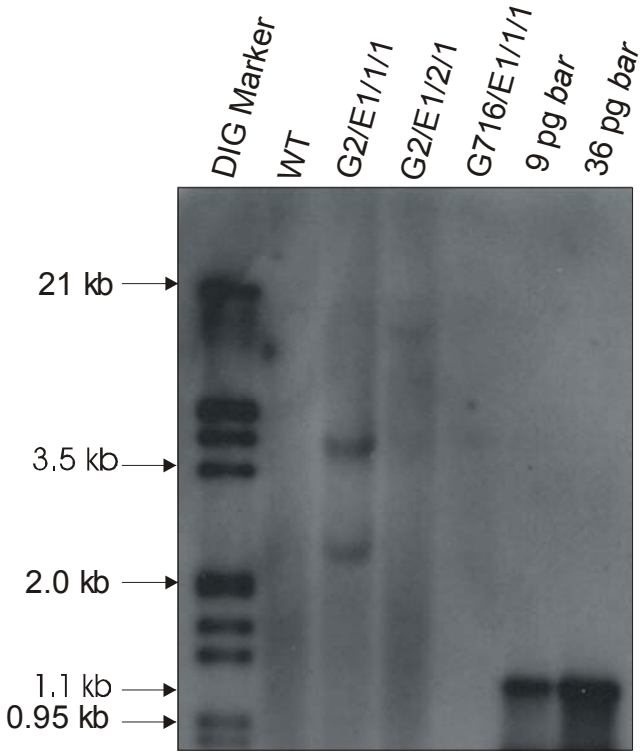

Fig (14): Southern blot hybridisation of genomic DNA digested with *HindIII* (T0). A DIG labelled *bar* gene fragment was used as a probe. As a positive control 9 and 36 pg *bar* fragment were applied.

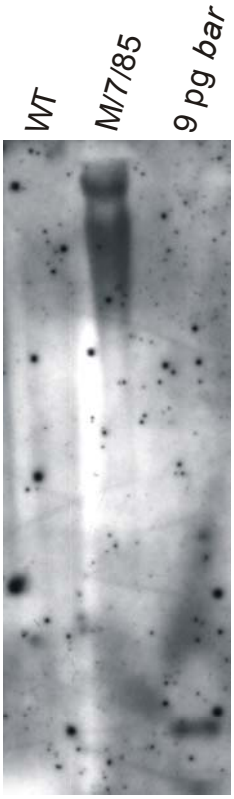

Fig (15) Southern blot analysis of a representative putative transformant (T0). 25 µg undigested DNA from clone M/7/85 were blotted to a nylon membrane and probed with the 1.1 kb *bar* gene (DIG labelled). As a positive control 9 pg *bar* fragment were used.

3.2.3 Analysis of the transformant progenies

3.2.3.1 PCR analysis of the transgenic progeny

Seeds of T0 plants were germinated in the greenhouse and DNA was isolated from young leaves of all plants that were germinated. In PCR analysis using specific primers for the *SFA8* gene, a DNA fragment of the expected size of 750 bp in length was amplified from the total DNA of the transgenic plants and from plasmid pGlsfa as a positive control (Fig 16). These DNA fragments were not detected in the DNA of untransformed plant (WT). Afterwards, the next generations (T2 and T3) were analysed by PCR to confirm the inheritance of the integrated genes in the progenies (Fig 17).

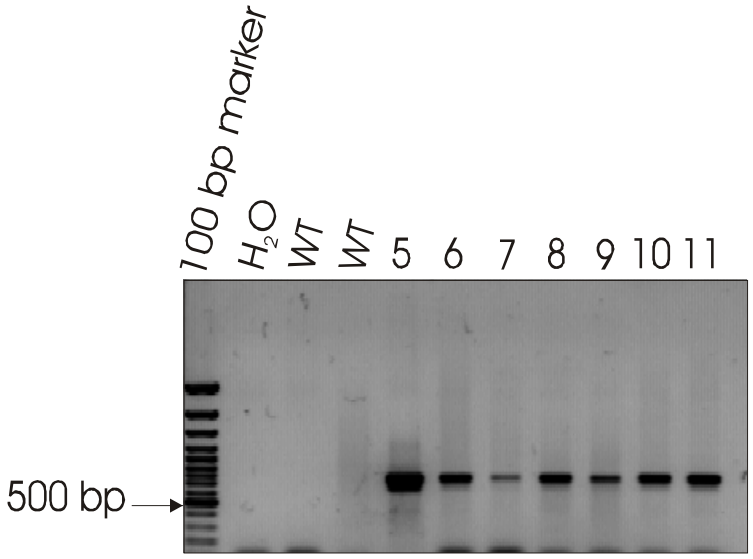

Fig (16): PCR analysis of transformed faba bean cv 'Albatross' (T1) of clones Abk1/5/1 (lanes 6-9) and Abk3/4/3 (lanes 10-11). Lane 5 is a positive control (pGlsfa plasmid). The size of the amplified fragment is 750 bp.

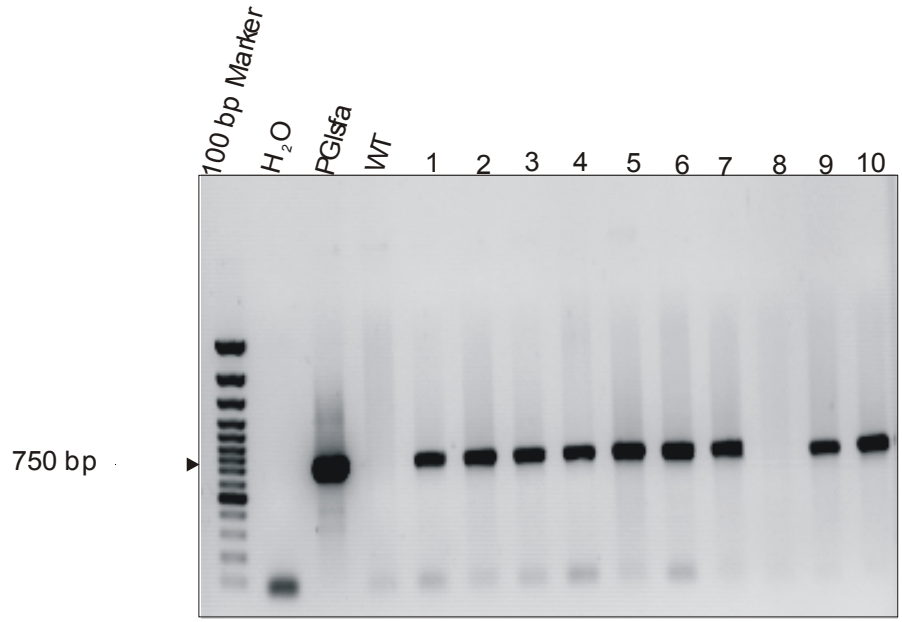

Fig (17): PCR amplification of the coding region of *SFA8* from genomic DNA isolated from transformed T3 progeny of clone G2/1/2 (lanes 1, 2 and 3), T2 plants of clone M/7/85 (lanes 4, 5, 6 and 10), clone ME1/2/1 (lane 7) clone MRE2/3/14 (lane 9) and T0 clone from Mfk2/1 (lane 8). The size of the amplified fragment is 750 bp

With respect to the co-transformation experiments the putative transformants (T0) or the progeny (T1) of the transgenic plants were PCR analysed for the integration of the T-DNAs. Putative transformants which were selected on PPT containing medium (the selectable marker of pGlsfa plasmid) were screened by PCR for the integration of both T-DNAs using primers for *SFA8* and *lysC* genes. The results obtained confirmed that only the *SFA8* gene was integrated into the plant genome. On the other side, the T1 from clones selected on kanamycin containing medium (the selectable marker in plasmid pAN109) were PCR analysed and it only the integration of the *lysC* gene was found (Fig 18). There is no evidence for co-integration of the *SFA8* gene in the same clone. The regenerated plants from callus clone Mfk2/1, which were selected on kanamycin and PPT showed no amplification signal neither for the *SFA8* nor the *lysC* gene (Fig 17). This confirms that this clone escaped the selection. The results obtained showed that the integrated foreign genes were transferred from only one plasmid and the transgenic plants contain either the *SFA8* gene or the *lysC* gene.

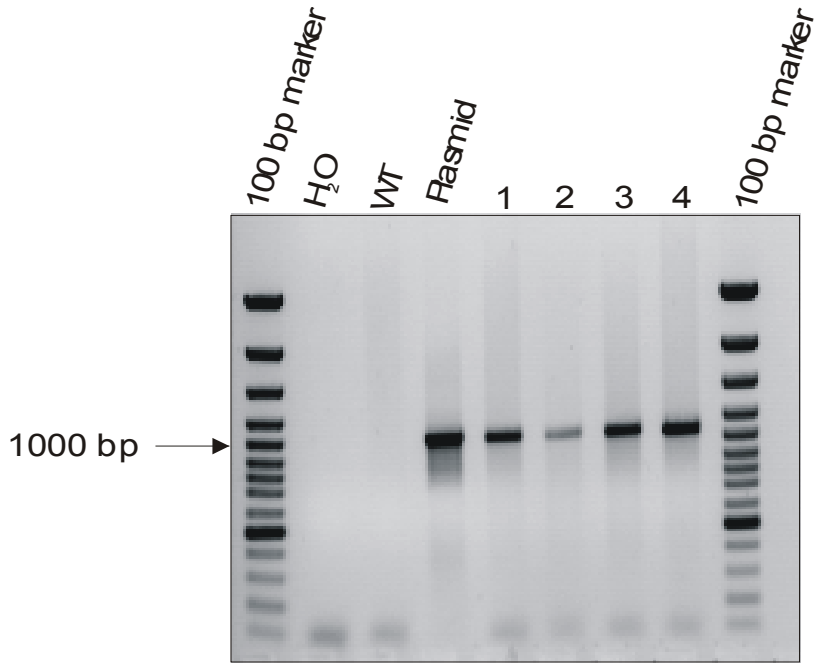

Fig (18) PCR analysis of the co-transformed progeny (T1) of the *Vicia faba* transgenic clone Mfk2/13 for the *lysC* gene (*AK*). The size of the amplified fragment is 1056 bp.

3.2.3.2 Expression of the marker gene

Expression of the phosphinothricin-resistance gene (*bar*) in the progeny of the transgenic plants (T1) was determined by the PAT enzyme (Fig 19) and leaf paint assay in the greenhouse (Fig 20). Transgenic clone G2/1/2 which did not express the phosphinothricin-resistance gene by either PAT assay or by leaf paint but which was shown by PCR to harbour the gene of interest (*SFA8* gene) until T4 and by southern blot (T1) analysis was also recovered (Fig 17, and 22).

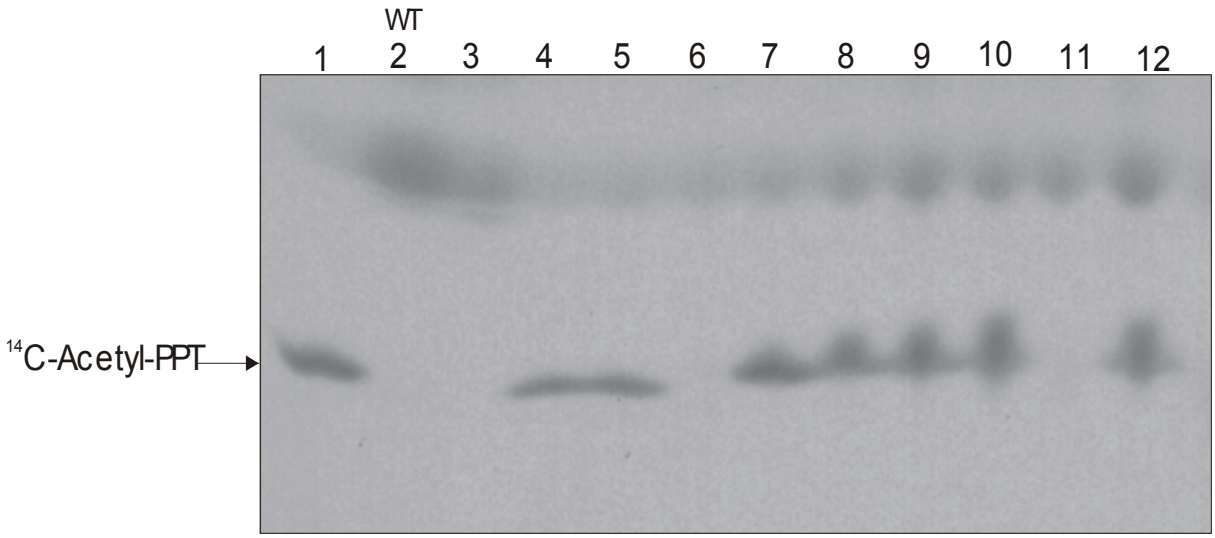

Fig (19) PAT enzyme assay for the progeny (T1) of two transgenic clones MfkE1/2/2 (lanes from 3 to 6) and clone ME1/2/1 (lanes from 7 to 12). Lane 2 is untransformed plant (WT) and lane one is a positive control.

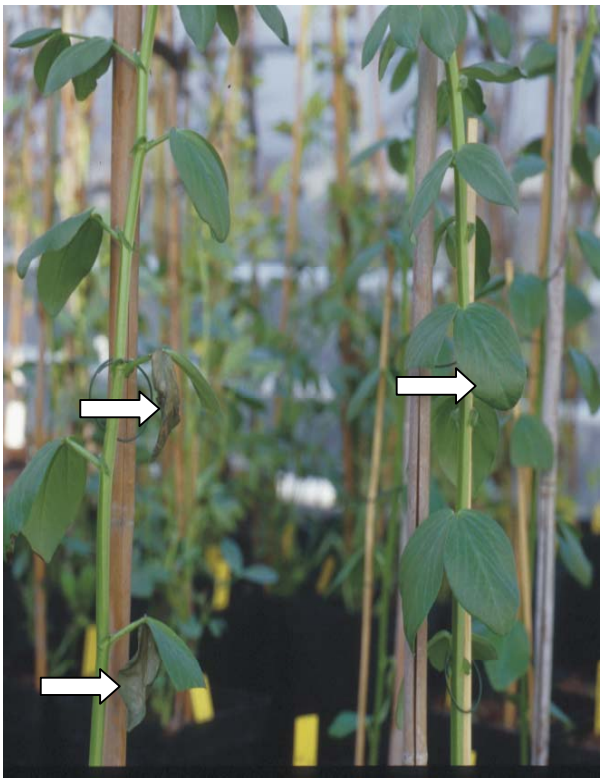

Fig (20) Herbicide (BASTA®) resistance in transgenic faba bean progenies. Leaflets (arrows) were painted with BASTA dilution in nontransformed plant (left) and transgenic plant (T2) expressing the introduced *bar* gene**.**

3.2.3.3 Southern blot analysis of the transgenic progeny

Southern blot analysis was used to prove the integration of the T-DNA in the plant genome. Plant genomic DNA was isolated form young leaves of transgenic progeny plants of the different transgenic clones. DNA digestion was performed with *BamHI* or *HindIII* and the fragments were subjected to Southern-blot analysis using 1.1 kb *bar* or *SFA8* (750bp) fragments (DIG labelled) as probes.

Digestion of the genomic 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. Fig (21) shows the expected hybridizing band at 3.5 kb.

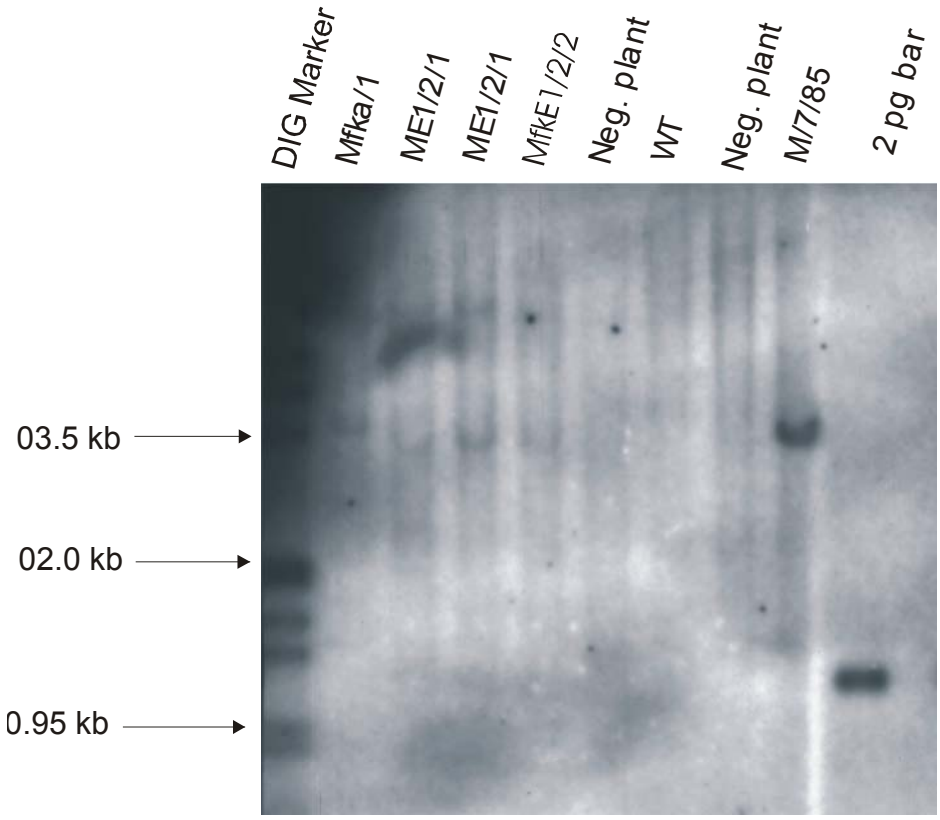

Fig (21) Southern blot analysis for the *bar* gene of the progeny of 3 clones (Mfka/1, ME1/2/1 and MfkE1/2/2), primary transformant clone M/7/85 and non-transformed faba (WT). Genomic DNA was digested with *BamHI.* As a positive control 2 pg *bar* fragment was used.

Plant genomic DNA was digested with *HindIII* and blotted to a nylon membrane in order to investigate the copy number of the transferred genes. A DIG-labelled 750 bp *SFA8*-fragment was used as a probe which hybridises with the T-DNA/plant-DNA junction fragments. Fig 22 shows the genomic blot of two lines, analysed as T1 generation (lines G2/1/2 and ME1/2/1). Line M/E1/2/1 contains a single copy of integrated T-DNA, whereas line G2/1/2 is harbouring two copies of T-DNA; no hybridisation signal was observed in the WT.

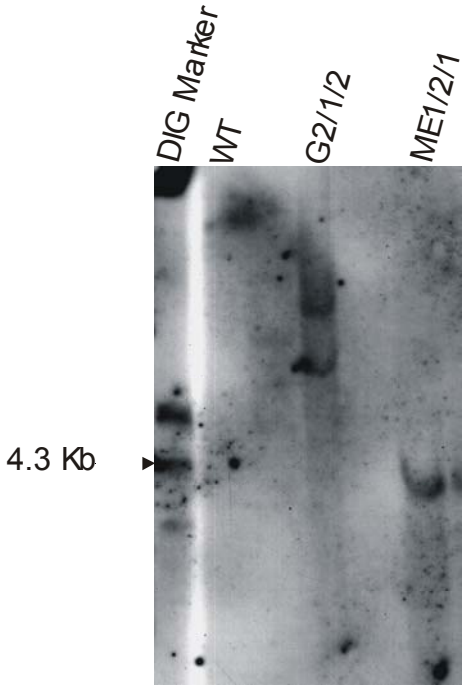

Fig (22) Southern blot analysis of *HindIII*-digested DNA, isolated from T1 plants of the lines G2/1/2 and ME1/2/1. DNA was hybridised with a 750 bp DIG labelled *SFA8* coding region.

3.2.3.4 Transgenic expression of *SFA8* gene

The expression of the *SFA8* transgene in transformed *Vicia faba* clones was studied by RT-PCR analysis and western blot. 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, which is driven by the seed specific legumin promoter. 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 (Fig 23). The leguminB4 gene transcript accumulates to high amounts in developing seed of *Vicia faba*. RT-PCR of *SFA8* results in the amplification of a fragment of 550 bp, lacking the intron sequence of 200 bps. 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.

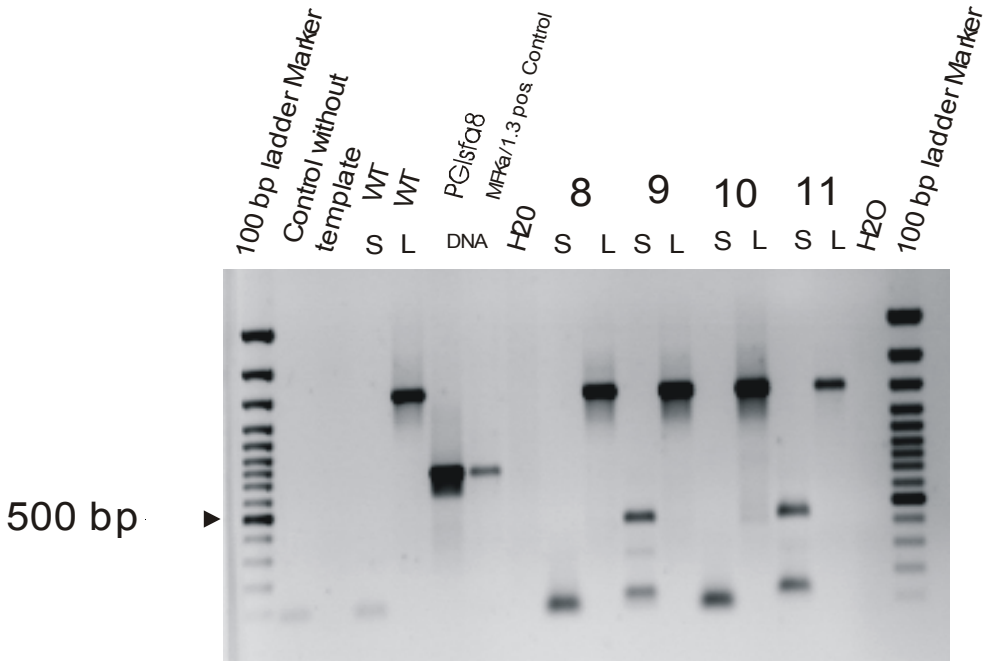

Fig (23): Representative RT-PCR analysis, for SFA8 (S lanes) and Legumin (L lanes, as internal control) transcription in the immature embryos (30 days after pollination) of T2 and T3 transgenic faba plants of clones MRE2/3/12 (lanes 8 and 10), MfkE1/2/2 (lanes 9) and clone Mfka/1 (lanes 11). *SFA8* transcript 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.

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 analyses 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 (Fig 24). No signals were observed in the faba bean wild type or in the negative plants (negative control). Some clones did not express the *SFA8* protein (clones G2/1/2 and M/7/85, the first had no expression of the *SFA8* gene neither at the RNA level nor the protein level, although 2 copies from *SFA8* gene were integrated in this clone Fig 22). On the other hand, in clone M/7/85 the *SFA8* transcript was detected by RT-PCR analysis (Fig 25) but there could no protein accumulation detected by western blot. This can be the result of a post transcriptional inhibition. In all cases where both RNA and protein were detectable, the presence of the *SFA8* protein was associated with the corresponding mRNA. Figure 25 shows the comparison of the transcripts of the two progenies of faba bean transgenic clones Mfka/1, that accumulated the *SFA8* protein and M/7/85 clone which did not accumulate the foreign *SFA8* protein. The *SFA8* transcript in the immature embryos of clone M/7/85 accumulated to different amounts. Representative results in Fig (26) showed the accumulation of the *SFA8* protein in different independent transformants of faba bean. It is clear that there is no considerable difference in the *SFA8*-protein accumulation between different clones.

The stability of the foreign protein in different generations was also a part of this study. Thus, SFA8 protein stability during different generations was analysed by western blot. Fig (27), shows that the accumulation of the *SFA8* protein was accumulated in plant Mfka/1.3.1 (T2) and its progeny (T3) at a uniform level.

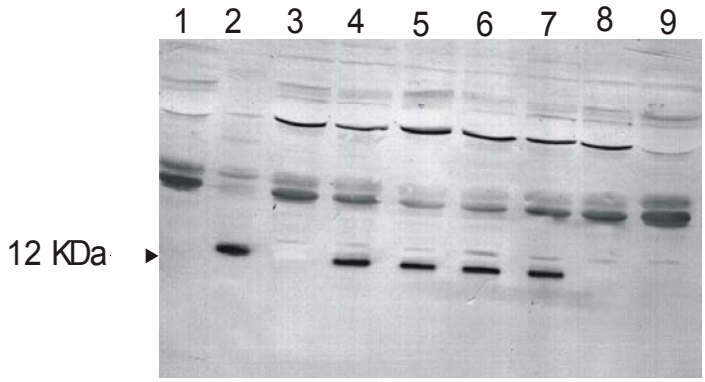

Fig (24): Western blot analysis of individual dry seeds (T2 generation) of clone Mfka/1. 50 µg of total seed SDS-soluble protein was separated in 15% SDS/PAGE, blotted to PVDF membrane and probed with antibody against sunflower albumin. Lanes 1, 8 and 9: untransformed Faba bean, lane 2: 26 µg of total soluble seed protein of sunflower (positive control).

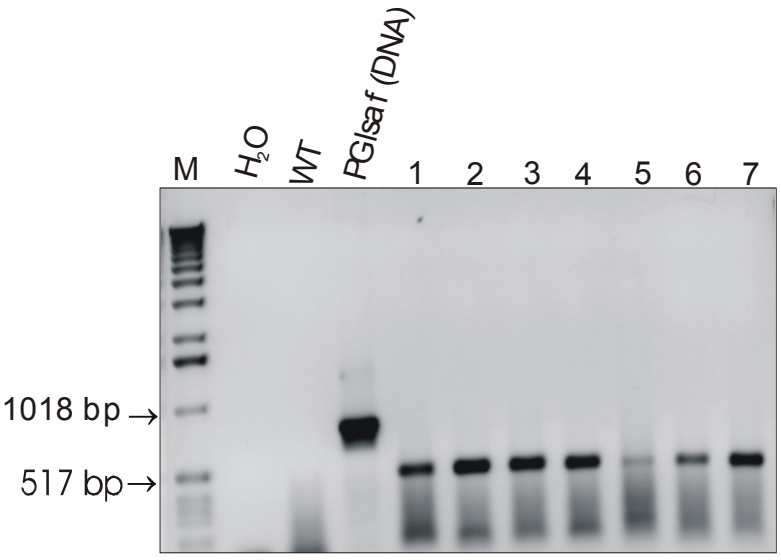

Fig (25): RT-PCR analysis of *SFA8* expression in immature embryos (30 days after pollination) of clones Mfka/1 (lanes 1, 2 and 3) and M/7/85 (lanes 4, 5, 6 and 7) progenies. RT-PCR of *SFA8* resulted in the amplification of 550 bp fragment (lacking of the intron sequence of 200 bp). M is DNA X marker.

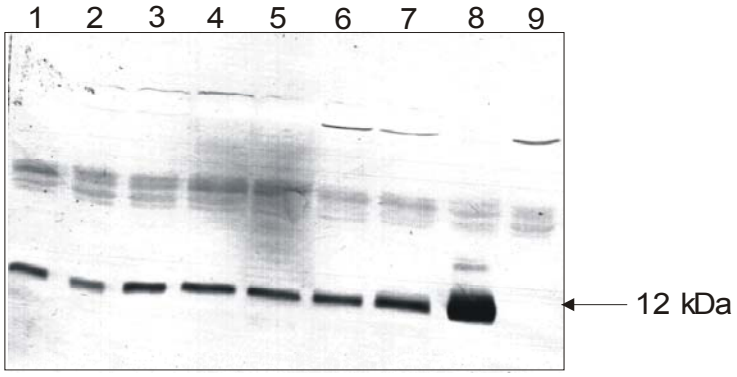

Fig (26): Western blot analysis of four independent transformant clones of *Vicia faba.* Each lane was loaded with 40µg total proteins of individual dry seeds of transgenic faba bean. Lane 1 is T3 of clone MfkE1/2/2, lanes 2 and 3 are individual seeds (T2) of clone MRE2/3/14, lanes 4 and 5 are individual seeds (T3) of clone ME1/2/1 and Lanes 6 and 7 are individual seeds (T3) of clone Mfka/1. As a positive control 26 µg of total extractable seed protein of sunflower were loaded (lane 8). Lane 9 is a negative control (Faba bean wild type).

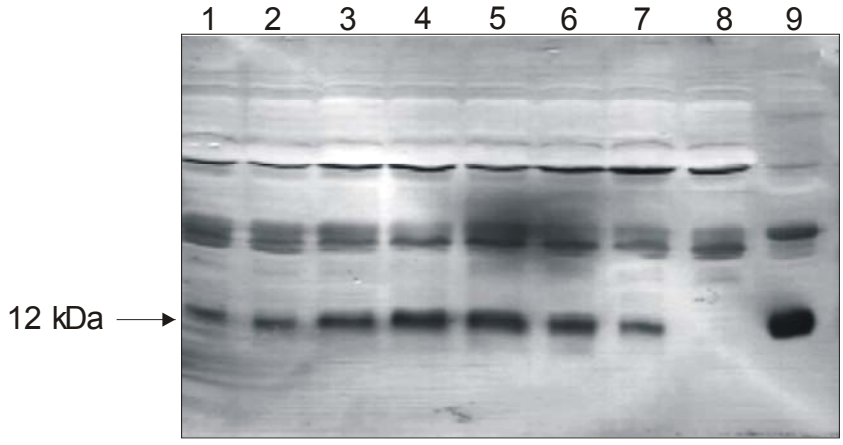

Fig (27): Western blot showing the accumulation of the *SFA8* protein in the T2 transformant of plant Mfka/1.3.2 (lane 7) and plant Mfka/1.3.1 (lane 6) and its progeny (T3) lanes 1, 2, 3, 4 and 5. Each lane was loaded with 80 µg of total extractable protein from mature seeds of transgenic *Vicia faba* and 40 µg of total extractable seed protein of sunflower (lane 9). Lane 8 is a negative control.

3.2.3.5 *SFA8* gene expression in transgenic embryos of the cultivar 'Albatross'

As soon as the transgenic clones derived from cv. 'Albatross' were recovered, the *SFA8* gene expression and its respective protein accumulation were monitored by RT-PCR and Western blot analysis. Figures 28 A and B represent the results of *SFA8* transcript detection by RT-PCR and the corresponding Western blot analysis in the cotyledons of immature embryos of T2 seeds of clone AB1/2/3. The levels of foreign protein which were detected by Western blot analysis (Fig 28) seem to be low; this is suggesting that the protein continues to accumulate until full maturity of the seeds. The results obtained demonstrate that the accumulation of *SFA8* protein was associated with the corresponding mRNA.

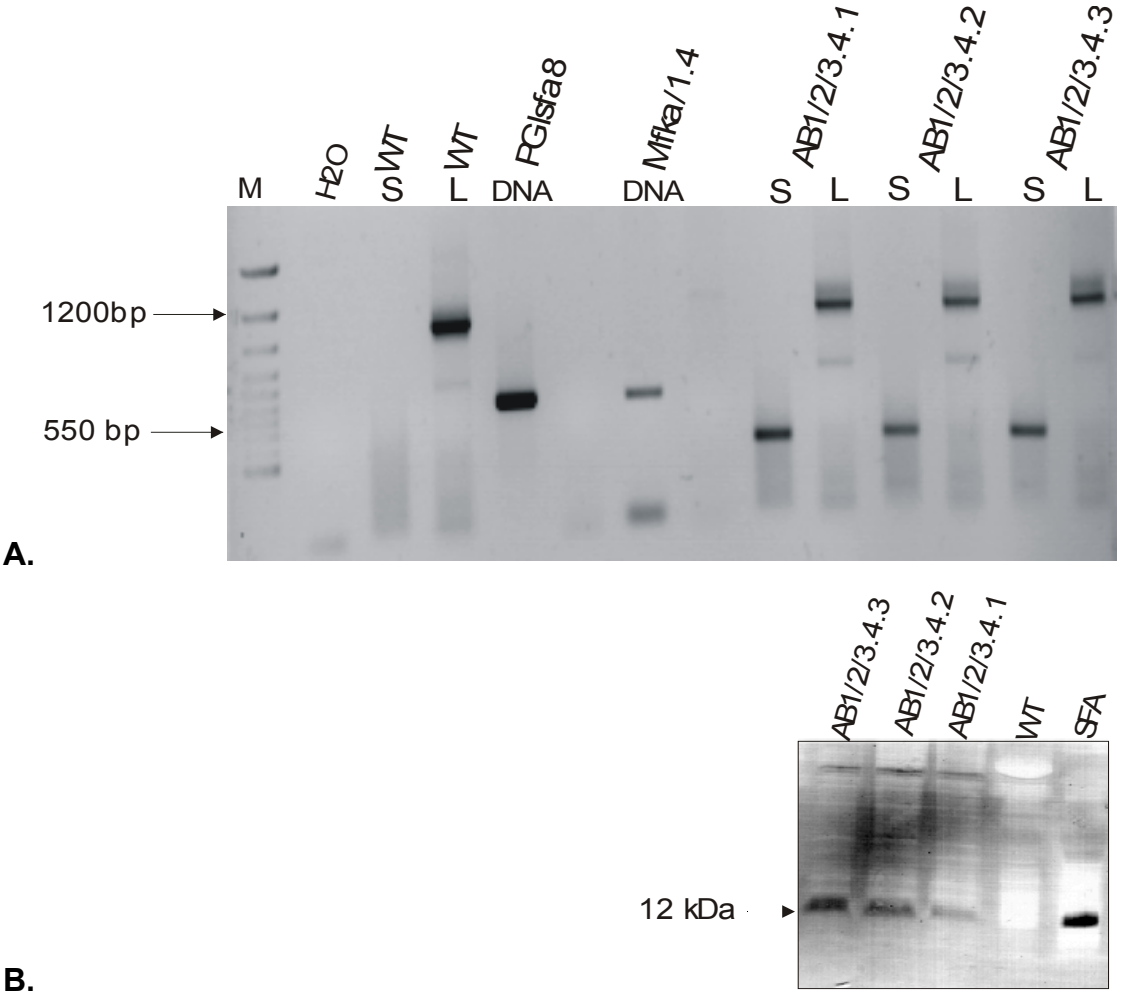

Fig (28 A-B): Molecular analysis of the transgenic faba bean T2 plants (cultivar Albatross) transformed with the pGlsfa plasmid. **A)** RT-PCR analysis for *SFA8* (S lanes) and leguminB4 (L lanes) transcription using total RNA samples isolated from immature embryos (30 days after pollination). *SFA8* transcript resulted in the amplification of a 550 bp fragment and legumin transcript resulted in the amplifications of 1200 bp in all samples tested. **B)** Corresponding western blot analysis; 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). Faba bean wild type (WT) was used as a negative control.

3.2.3.6 Transgenic expression of *lysC* gene

The expression of the *lysC* gene was analysed by western blot using a polyclonal antibody specific for the *lysC* gene product, a bacterial aspartate kinase insensitive to feedback control (AK). This gene was driven by a seed specific promoter (phaseolin promoter) and the product of this gene was targeted to the cotyledons of the developing embryo (Böttinger et 2001). Fig (29) shows that the 47 kDa aspartate kinase was detected in the seeds of (T1) of clones Mfk2/13 and Mfk2/3. No signal was detected in the negative control (wild type).

Table 7 summarises the results obtained from molecular and biochemical analyses from all transgenic clones.

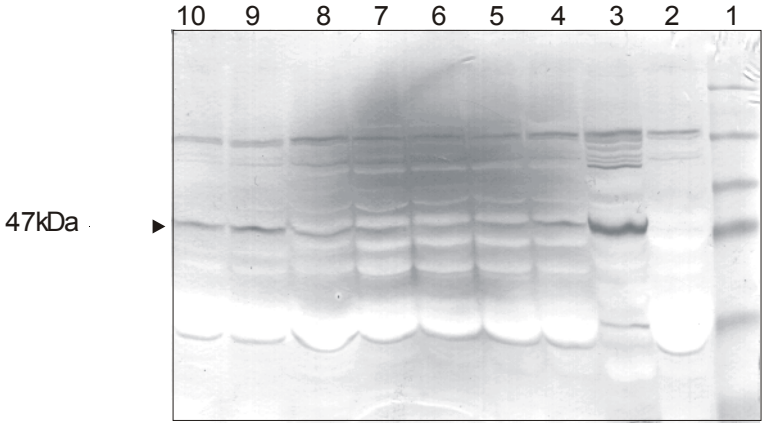

Fig (29) Western blot analysis from protein extracted from mature seeds of transgenic faba bean clones Mfk2/13 (lanes 4,5,6 and 7) and Mfk2/3 (lanes 8, 9 and 10). About 85 µg SDS total extractable protein from individual mature seeds were loaded on each lane. Lane 3 is transformed pea with *lysC* gene (which transformed with another vector) as a positive control. Lane 2 is a wild type of Faba bean as a negative control and lane 1 is pre-stained protein marker. The 47 kDa protein was detected only in the transgenic Faba bean and used as the positive control.

Table (7): *Vicia faba* transgenic clones and analysis of the progenies by (i) PAT assay, (ii) leaf paint (BASTA), (iii) PCR, using either *SFA8* or *lysC* primers, (iiii) Southern blot analysis using either *SFA8* or *bar* as probes, (V) Western blot analysis for *SFA8* and *lysC* genes. Segregation has been estimated by PCR analysis and (or) leaf paint test.

Cultivar	Clone	Transformation method	Plasmids/ transferred genes	Transgenic analysis							Segregation (No. of the seeds tested)	
				PAT	BASTA	PCR	Southern blot (*bar/SFA8*)	Western blot	Character	Clone stage	T1	T2
Mythos (M)	***ME1/2/1***	Immature embryo axes	pGlsfa/ *SFA8-bar*	+	+	SFA8	+	*SFA8*	Normal	T4	5 :1	9 :0
	Mfka/1	Immature embryo axes	pGlsfa and pAN109/ *SFA8 - bar*, *lysC - nptII*	+	+	*SFA8*	+*	*SFA8*	Normal	T4	4 :3	7 :0
	MfkE1/2/2	Immature embryo axes	pGlsfa and pAN109/ *SFA8 - bar*, *lysC* - nptII	+	+	*SFA8*	+*	*SFA8*	Normal	T4	4 :3	8 :2
	MRE2/3/12	Mature embryo axes	pGlsfa/ *SFA8-bar*	+	+	*SFA8*	nd	*SFA8*	Normal	T3	9:4	nd
	M/7/85	*de novo*	pGlsfa/ *SFA8-bar*	+	+	*SFA8*	+*	-	Normal	T3	3:2	4:2
	M/7/39	*de novo*	pGlsfa/ *SFA8-bar*	nd	+	*SFA8*	nd	*SFA8*	Weak	T1	3:1	nd
	M/1/339	*de novo*	pGlsfa/ *SFA8-bar*	+	+	*nd*	nd	nd	Abnormal (No seeds)	T0	nd	nd
	Mfk2/3	*de novo*	pGlsfa and pAN109/ *SFA8- bar* , *lysC* - nptII	-	-	*lysC*	nd	*lysC*	Abnormal pods	T1	nd	nd
	Mfk2/13	*de novo*	pGlsfa and pAN109/ *SFA8 - bar,* ysC - *nptII*	-	-	*lysC*	nd	*lysC*	Normal	T1	nd	4:1
	Mfk1/35	*de novo*	pGlsfa and pAN109/ *SFA8- bar* , *lysC - nptII*	nd	nd	*SFA8*	nd	nd	Normal	T0	nd	nd
Giza 2 (G2)	G2/1/2	*de novo*	pGlsfa/ *SFA8 - bar*	-	-	*SFA8*	++	-	Normal	T4	2:0	4:2
	G2/1/23	*de novo*	pGlsfa/ *SFA8 - bar*	+	+	*SFA8*	nd	nd	Abnormal (No seeds)	T0	nd	nd
Albatross (A)	AB1/2/3	Immature embryo axes	pGlsfa/ *SFA8 - bar*	+	+	*SFA8*	nd	*SFA8*	Normal	T2	1:1	3:0
	Abk1/5/1	Immature embryo axes	pGlsfa and pAN109/ *SFA8- bar* , *lysC - nptII*	nd	+	*SFA8*	++**	nd	Normal	T2	5:0	nd
	Abk3/4/3	Immature embryo axes	pGlsfa and pAN109/ *SFA8- bar* , *lysC - nptII*	nd	-	*SFA8*	++++**	nd	Normal	T1	nd	nd

* contains at least one copy

** date not shown

3.2.4 Amino acid analysis of seed proteins from transgenic faba bean

The amino acid compositions of Faba bean seed meals from wild type and transgenic plants were analysed in order to estimate the level of methionine and cysteine content. Fig (30) presents the amino acid profile of seed proteins extracted from control plants (wild type) and one of the transgenic clones (Mfka/1) which accumulated the foreign protein at detectable levels (see Fig 26-27). The transgenic seeds contain methionine at levels of 0.76 % of total crude protein. This level represents an increase of 15.1% for the total methionine found in the seeds as compared to the wild type (0.66% methionine of the crude protein). The cysteine level increased by 23% in comparison to the wild type. The methionine level of other transgenic lines of T2 and T3 generations was analysed as well. It was found that there were minor changes in the levels of the methionine content in the seed meal of the transgenic clones as compared by the wild type of faba bean and the correspondent negative plants as well with 8% at maximum increasing in the methionine level (data not shown).

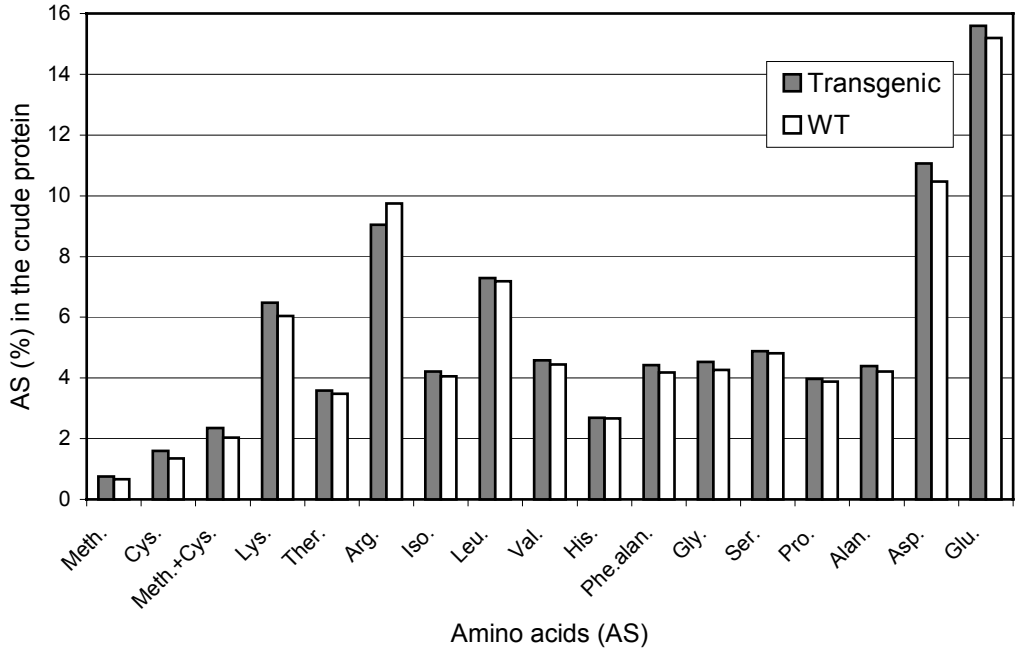

Fig (30): Amino acids (%) composition of Faba bean seed meal from wild type and transgenic plants which grown in greenhouse.

4. Discussion

The main goal of this study was the improvement of the nutritional value of Faba bean through gene transformation based methods. This implied the development of a reproducible and stable transformation protocol with a broad applicability on Elite cultivars of Faba bean.

As soon as transformed plants with the respected genes of interest were obtained, the focus was on analysing both the expression and the stability of the transferred genes in subsequent generations as well as on the modification of the overall protein composition.

4.1 Transformations methods

There is only a single report on *Vicia faba* cv 'Mythos' transformation where fertile transgenic plants were recovered (Böttinger et al. 2001). This approach was based on plant regeneration from internodal stem segments derived callus (meristem lacking tissues). This protocol has been tested in this 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*. Calli 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 another and from one type of explants to the other type. The frequencies of callus induction under selective conditions were between 4.3-31.59%. Shoot bud regeneration from the selected calli ranging between 6.6% with Giza 716 and 0.9% with Giza 429 cultivars (Table 2). Regeneration capacity from the selected callus is very low as expected, in contrast to model plants like Solanaceae. This result is in the same range as previous work done by Böttinger et al. (2001) (regeneration frequency was between 2.7% and 6%), or from Tegeder et al. (1995) with *Vicia faba*. The problem is 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). The recovery of seed producing putative transformed plants under these circumstances took about 16-24 months, a

considerably long period. The main constraints in this protocol were the poor regeneration ability *via* callus phase, the reduced fertility and the high percentage of phenotypic abnormalities in the regenerated plants (see 3.1.1.1). Possibly this is due to the long cultivation time *in vitro* (around 7-16 months) McClintock (1984).

Transformation protocols based on *de novo* regeneration of shoots from dedifferentiated cells have rarely been applied to produce transgenic grain legumes. Puonti-Kaerlas et al. (1990) obtained transgenic pea (*Pissum 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 Puonti-Kaerlas et al. reported chromosome doubling (tetraploidy) in their transgenic pea. There are also successful transformation systems which 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). Except the mentioned plant species there is no other transformation protocol based on shoot regeneration either by somatic embryogenesis or organogenesis from dedifferentiated cells (callus) which proliferated from explants lacking meristems published. This confirms the fact that the major limitation of grain legumes transformation in general is the requirement for effective *in vitro* regeneration system as compared to model plants. 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 in this case. The major success in legume transformation was achieved by methods based on transformation of the pre-existing meristems on the embryo axes, shoot tips, nodal explants or cotyledonary nods. 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), in chickpea (Krishnamurthy et al. 2000; Kiesecker 2000), *Lupinus angusitifolius* (Pigeaire et al. 1997) and soybean (Yan et al 2000). 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* transformation for *Vicia faba* via meristematic cells was proven and optimized. 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 transgenic *Vicia faba* in combination with *Agrobacterium tumefaciens*. Seven transgenic clones were produced from two cultivars (i.e. Mythos and Albatross). The transferred genes were shown to be stably inherited and expressed in the next progenies of transformed plants till T4. Transformation frequencies were ranging from 0.15% to 2.0%.

The actual transformation system 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 manner the possibility of somaclonal variation has been reduced to the minimal level. This transformation system was an adaptation of the protocol which previously was reported by Schroeder et al. 1993 in *Pisum sativum*. This transformation system allowed to obtain the primary seeds bearing transformants (T1) to be recovered within approximately 9-10 months. Table 8 presents a summary of the results which were obtained with both transformation methods (*de novo* regeneration and direct shoot organogenesis). These results confirm that both transformation strategies have the potential to produce transgenic plants. It is clear however, that embryo axes transformation methods are more efficient and faster for the production of fertile transgenic plants when compared to the *de novo* regeneration protocol.

Table (8): Comparison of the *Vicia faba* transformation protocols

Cultivars	*de novo* regeneration protocol				Direct shoot organogenesis			
	no. of expants (meristem lacking tissues)	Reg.	T0 Plants (Selected on PPT)	T1 Plants	No of explants (embryo axes)	Reg.	T0 Plants (Selected on PPT)	T1 Plants
Mythos	3087	12	7	3	461	26	26	4
Albatross	0	0	0	0	1047	14	14	3
Giza 2	2237	2	2	1	81	2	2	0
Giza 429	2156	1	0	0	132	0	0	0
Giza 716	749	2	1	0	123	3	3	0
Giza blanka	738	0	0	0	123	0	0	0

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. Thus, it can be recommended that the regenerated shoots should be selected for at least 3-4 months before recovering the plants by micro-grafting. Short-term selection in principal increases the possibility for chimeric plants, which are mainly useless. 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 and McCabe 1992; Sato et al. 1993) or with *Agrobacterium*-mediated transformation (Bean et al. 1997; Grant et. al. 1995).

Christou and McCabe 1992 obtained more chimeras than clonal transformants after transformation of multicellular tissues of soybean by electric discharge particle acceleration. They reported that not all the chimeric transformants transmitted the introduced genes to the next generations. Their results indicate, whilst monitoring the GUS activity in the transgenic tissues of the primary transformants, that the majority (not all) of germ-line transformation events which were derived from plants with stem segments showing an extending of GUS activity from epidermis through cortex to the pith resulted in the recovery of transgenic progeny. 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.

The differences between the presented transformation system and the protocol which was reported by Böttinger et al. (2001) are the starting materials (mature and immature embryo axes vs. epicotyl segments) and the shorter time for *in vitro* culture (from 9-10 months until recovering of T1 generation vs.> 12 months for *in vitro* culture). The morphological and cytological abnormalities which were reported by Böttinger et al. 2001, were not observed in the present system.

The direct gene transfer to the mature embryo axes of the cultivar *Albatross* was tested in this study as well. In order to optimise the direct gene transfer system using particle bombardment (PDS 1000 He), different conditions and explants (embryo axes, cotyledonary nodes and cotyledons) were tested. The recovery of transgenic offspring through this type of transformation and regeneration needs L2-layer transformation in order to achieve germ line transformation (Satina et al. 1940). The regenerated shoots were screened for GUS activity (reporter gene) rather than selection for transgenic shoots. The reason is that selection of the transformed sectors in the transformed explants is risky as reported by McClean and Grafton 1989.

The biolistic approach was established particularly for recalcitrant plants like grain legumes and cereals (i.e. Christou 1990/soybean; Russel et al. 1993, Aragao et al. 1993 and 1996 /common bean; Brar et al. 1992 and 1994/ peanut). Up to date however, there is no published report on Faba bean transformation *via* direct gene transfer based methods reflecting the difficulties to recover a clonal transformed Faba bean with these approaches.

With all the conditions and explants tested, only chimeric shoots were regenerated, as judged by the amount and distribution of the GUS expression in the stems of the regenerated shoots after bombardment. Therefore, the probability that no-germ-line transformation has been occurred is rather high. Therefore no detailed molecular investigations with this material was performed.

4.2 *Using of bar* gene as a selectable marker

The assessment of the transformation by these methods was based largely on the nopaline synthase-phosphinothricin acetyl transferase (*bar*) gene expression, which confers resistance to phosphinotricin (PPT, the active ingredient of the commercial nonselective herbicide BASTA®). PPT is an inhibitor of glutamine synthase in plants (Krieg et al. 1990). The *bar* gene encodes the enzyme phosphinothricin acetyl transferase (PAT), which catalyses the PPT and yielding an inactive product, N-aceyl-PPT (De Block et al. 1987). The preliminary experiments showed that using PPT at concentrations of 2 mg/l totally inhibit the callus growth from untreated (control/wild type) epicotyl and leaf explants (*de novo* regeneration protocol). The same concentration of PPT has suppressed any growth or regeneration from the untreated embryo axes and the explants subsequently died. It could be pointed out that removing any callus growth from the regenerated shoots on selective medium was important for further distinguishing between clonal and chimeric transformants and facilitating direct uptake of the PPT as described by Bean et al. 1997. This basal callus could be *bar* resistant and detoxifying the PPT and thus protecting the regenerated shoots from the action of PPT. Also, the regenerated shoots must be selected for 4-6 months in order to avoid a high percentage of chimeric plants.

Successful expression of the bar gene was confirmed and monitored in the putative transgenic plants and the next sexual generations, by assaying PAT activity and performing the leaf-painting test. These results proved the suitability of using the *bar* gene as an effective selectable marker in *Vicia faba* transformation. On the other side the use of neomycin phosphotransferase (*nptII*) which confers kanamycin resistance for selection and identification of transgenic shoots was found to be ineffective (data not shown). This is because the untreated embryo axes (control) and the regenerated shoots are able to grow on comparably high concentrations of kanamycin (100mg/l).

PPT has been used as a selective agent successfully to obtain fertile transgenic plants such as in pea (Schroeder et al. 1993; Bean et al 1997), common bean (Russel et al. 1993), *Lupinus angustifolius* (Pigeaire et al. 1997), subterranean clover

(Khan et al. 1996) and in wheat (Sorokin et al. 2000) with different concentrations depending on the species.

4.3 Co-transformation

In co-transformation experiments we used two physically independent binary vectors, EHA105/pGlsfa which are carrying the coding sequence for the methionine rich sunflower 2S-albumin (*SFA8*) gene under a seed specific promoter (LeB4) in combination with the selectable marker *bar* gene. The second vector is EHA101/pAN109 harbouring a mutant *lysC* gene, coding for a bacterial aspartate kinase which is insensitive to feed-back control by threonine in combination with *nptII* as a selectable marker. Co-transformation by a double *Agrobacterium* infection was performed by both presented transformation systems (i.e. embryo axes transformation and by *de novo* regeneration transformation protocol/Böttinger et al. 2001). The transformation events were selected by two different approaches, **i)** selection for only one marker (PPT or kanamycin), afterwards the transgenic plants were screened for the presence of the second T-DNA; **ii)** double selection using kanamycin and PPT at the same time.

In respect to the co-transformation of the embryonic axes *via Agrobacterium* mediated gene transfer, the selection was done only for the *bar* gene. This decision was made because of the high level of tolerance of the embryo axes and the regenerated shoots to kanamycin selection. Co-transformants were screened by PCR analysis for both T-DNAs and occasionally by western blot analysis.

There was no transgenic clone which showed the integration of both T-DNAs. Only transgenic plants with one of the T-DNAs were generated, possibly as consequence of the low percentage of the regeneration and subsequently low transformation frequencies. Calli clones expressing resistance to both selectable markers were recovered.

Characterisation of the co-transformed events were reported in many studies specially with model species such as *Arabidopsis* and *Solanaceae* to introduce linked

and unlinked genes into plants, mainly by direct gene transformation (Damm et al. 1989/*Arabidopsis*; Tagu et al. 1988/*Petunia hybrida*; Christou and Swain 1990/Soybean cell culture; Aragao et al. 1996/common bean). *Agrobacterium*-mediated transformation has been applied with rapeseed (de Block and Debrouwer 1991; Daley et al 1998), *Arabidopsis* (De Buck et al. 1998) and tobacco (de Neve et al. 1997; Komari et al. 1996).

4.4 Integration of the foreign genes and progeny analysis

The present study showed the possibility of recovering transgenic faba bean plants using different methods. Many transgenic clones were recovered from 3 cultivars (Mythos, Albatross and Giza 2). For confirming the integration of the foreign genes into the plant genomes, Southern blot analysis is the method of choice. By Southern blot it is possible to identify independent transformation events. Also, PCR analysis is required specially for screening the transgenic progenies for studying the segregation of the introduced genes using specific primers, which could amplify specific fragments (foreign genes) in the transformants' genomes. During the research, expression of the introduced genes i.e., *bar* gene (by PAT assay and herbicide BASTA® leaf painting test, see 4.2), *SFA8* and *lysC* genes by RT-PCR and western blots were studied (see below).

Southern blots of undigested and digested DNA with restriction enzymes *HindIII* or *BamHI* subsequently probed with the *bar* or *SFA8* inserts, demonstrated integration of the introduced genes in the plant genome (see Fig.14, 15, 21, and 22). Primary transformants (T0 plants from 2 cultivars 'Giza 2 and Giza 716', regenerated from embryo axes) which were analysed by Southern blot, showed that at least one copy of the *bar* gene was integrated into the plant genome (Fig 14), taking into account that these plants did not express tolerance to the herbicide (BASTA®) leaf paint test. It is noteworthy to state that in further analysis of the mentioned clones in the next generations (T1) it was confirmed that these clones were chimeras. This can be due to the instability of the integrated genes in their progenies. Finally these clones were ignored. Southern blot hybridisation in T1 plants has confirmed the integration of the genes of interest into the plant genomes which were resistant to herbicide BASTA®

and also in the line G2/1/2 which did not express tolerance to BASTA (Fig 21, 22). The *BamHI* digest shows the expected 3.5 kb band, bands with higher molecular weight than 3.5 kb were also detected. This can be explained by partial digestion of the genomic DNA (Fig 21), since DNA digestion with *HindIII* shows the clear copy number of integrated T-DNA (Fig 14 and 22).

The *Vicia faba* genome is considered as being one of the largest genomes in the plant kingdom (13.33pg/C). The chromosome size and DNA content of *Vicia faba* is about double that of the other *Vicia* species (Raina and Ogihara 1995), this phenomenon caused difficulties in the detection of hybridisation signals, especially for the determination of the integrated copy number, as at least 25 µg/digested DNA is required to detect a single integration of the *bar* gene (1 pg).

The regenerated plants from PPT or Kanamycin selection, which were obtained from different transformation systems and showed no phenotypic aberrations were fertile. The next progenies revealed that the foreign genes were transmitted to the offspring. The inheritance data of the transgenic clones were studied, and it was observed that a stable inheritance with segregation patterns through multiple generations had occurred. Several lines were advanced for three generations of self fertilization with no loss of the transferred genes.

Due to the low number of seeds which were produced by the transgenic plants a statistical analysis has not been done. In some clones the segregation did not show exactly the Mendelian rate of 3:1 (presence: absence) for the inheritance of a single transgenic locus after self pollination. The segregation ratio was estimated by PCR amplification for *SFA8* or *lysC* genes and (or) determination of the *bar* gene expression by leaf paint test (Table 7).

These experiments have demonstrated that the foreign genes have been delivered into faba bean *via* either transformation of pre-existing meristems (embryo axes) or transformation of the epicotyl explants (which are lacking of meristems), callus selection and finally regeneration of the transgenic plants (*de novo* regeneration protocol).

4.5. Gene of interest expression (*SFA8*) and protein analysis

The main aim 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 (Kortt et al. 1991), and the a mutated *lysC* gene coding for the a bacterial aspartate kinase, which was desensitised to feed back inhibition by lysine and threonine were transferred to Faba bean. The direct combination of both genes by co-transformation, *SFA8* as a sink for sulphur containing amino acids and *lysC* as source for free methionine in one double transformant should be investigated. This approach was impossible due to the low percentage of the regeneration of the transgenic plants. Therefore, only transgenic plants with either the *SFA8* gene or with the *lysC* gene were regenerated and analysed.

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 promotor (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 (BNA) 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 amplification of *SFA8* gene shows weaker signals than that observed from the endogenous legumin gene amplification.

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 sunflower 2S albumin was detected by western blots in six 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 (Pickardt et al 1995, 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. Taking into account that *SFA8* includes about 7-8% of the total sunflower seed protein, the amount of the foreign protein was estimated to be in between 0.4-0.8% of the total soluble seed protein in transgenic *V. faba*. The accumulation of the sunflower 2S albumin was not high enough to change the amino acids pattern significantly. It was found that these transformants did not exhibit a significant increase in the methionine content of the salt soluble seed protein. This indicates that the transcript message is inefficiently translated or the introduced protein incorrectly processed. A possible reason for this phenomenon was described by Molvig et al. 1997 in transgenic lupins, where it was found that in transgenic plants expressing the *SFA8* protein, probably simultaneously other changes in the composition of the seed protein fractions had occurred. For example, a reduction in the level of a native protein which contains considerable amounts of methionine and cysteine such as conglutin δ could cause the insignificant change in the total sulphur containing amino acids in the seed meal of the transgenic plants.

Production of transgenic plants expressing genes coding for sulphur rich proteins e.g. the Brazil nut 2S albumin *BNA* (Sun et al. 1987) or the sunflower 2S albumin (*SFA8*), has been repeatedly reported (see introduction). From surveying the published reports, there were 20 research papers published, in which genetic transformation based methods were applied to improve the protein quality using BNA or *SFA8* genes. In the BNA case, the modified protein was stably expressed with significant amounts in different plants (e.g. tobacco/Altenbach et al. 1989; *Arabidopsis*/Conceicao et al. 1994; Canola/Altenbach 1992; *Vicia narbonensis*/Pickardt et al 1995; Saalbach et al. 1995a/1995b; Soybean/Jung et al. Pioneer Hi-bred. Inc. USA 1997; *Phaseolus vulgaris*/Aragao et al. 1999). On the other hand, Guerche et al. (1990), De Clercq et al. (1990) and Tu et al. (1998) failed to obtain the accumulation of the Brazil nut methionine rich protein altering the amino acid composition in different plants. After publishing the allergenic effects of the Brazil nut methionine rich protein (Nordlee et al. 1996), the research moved to use the sunflower 2S albumin gene (*SFA8*) instead.

Accumulation of the *SFA8* protein up to 0.3% of soluble leaf protein was achieved in transgenic tobacco with the CaMV35S/*SFA8* construct (Tabe et al. 1993). The same research group in 1995 reported the accumulation of this foreign protein in leaves of transgenic alfalfa to up to 0.1%, when the *SFA8* gene was driven by *atsA*1 promotor

from *Arabidopsis.* In transgenic subterranean clover, Khan et al. 1996 reported the accumulation of the *SFA8* protein to up to 0.3% in the edible leaf protein. Christiansen et al. 2000 in transgenic *Trifolium repens* and Wang et al. 2001 in transgenic Tall fescue obtained accumulation of the foreign protein between 0.1 and 0.2%. These contents, however, were not sufficient to alter the methionine level significantly. Wang et al. 2001 mentioned that the useful level of *SFA8* protein for a significant impact on ruminant diet would be in the order of approximately 4%. To date the only successful report which applied this approach was in transgenic lupins, as reported by Molvig et al. 1997. Approximately 5% of the soluble seed protein of the transgenic lupins was *SFA8* when the gene driven by the seed specific promoter from a pea *vicilin* gene. This level of foreign protein was associated with increasing the methionine in the seed proteins by 94% more than the wild type but with an unexpected 12% reduction in cysteine. The same research group had reported the recovery of transgenic pea and chickpea with the same construct and the accumulation of the foreign protein at a detectable amount in the seed proteins (unpublished data).

With respect to the *lysC* transformants, two clones (Mfk2/13 and Mfk2/3) which showed the integration of the *lysC* gene under the control of the seed specific phaseolin promotor were subjected to Western blot analysis. The 47KDa aspartate kinase was detected in the seed proteins. This result is in accordance to the previous work of Böttinger et al. 2001 and Karchi et al. 1993.

5. Conclusion and outlook

In the present study, different transformation systems were tested with different selected *Vicia faba* cultivars. The major outcome of these experiments is an efficient *Agrobacterium* transformation protocol by means of direct shoot regeneration after transformation of meristematic cells (mature and immature embryo axes). The use of this new developed transformation protocol enabled to overcome many problems that stemming from callus phases which implying a higher risk of somaclonal variation.

The transformation experiments were performed using either EHA 105/pGlsfa carrying the *SFA8* gene under a seed specific legumin B promoter and the selectable marker gene *bar*, or in combination with EHA101/pAN109 which harbouring *lysC* gene under the seed specific phaseolin promoter and the selectable marker gene *nptII*. Fifteen transgenic clones were recovered from 3 different Faba bean cultivars (*Mythos*, *Albatross* and *Giza* 2) carrying either the *SFA8*/*bar* or the *lysC*/*nptII* genes. Southern blot hybridisations demonstrated that at least one copy of the T-DNA was stably integrated into the plant genome. The inheritance of the transgenes was studied by means of PCR analysis until the T3 generation.

In most of the recovered transgenic clones the foreign genes were active as predicted from the expression of the selectable marker *bar* gene (PAT assay and herbicide BASTA® leaf paint test). The expression of the genes of interest *SFA8* and *lysC* genes was confirmed by RT-PCR and Western blot analysis. This study demonstrates that the transgenic expression of *SFA8* gene in *Vicia faba* leads to accumulation of the sunflower methionine rich protein at detectable levels with respect to total salt soluble seed proteins. In the transgenic Faba bean containing the *lysC* gene, the corresponding 47 kDa aspartate kinase was detected in the transgenic seed proteins.

For the future approaches, combinations of the *SFA8* and *LysC* genes could be possible *via* crossing of the *SFA8*-transgenic plants and *lysC*-transgenic plants in order to be in a position to embark on meaningful improvement of the protein quality of the *Vicia faba*. Major target for the transgenic Faba bean plants is the environmental risk assessment of the products which derived from the transformants

this is by screening the plants for the presence of bacterial *nptIII* to investigate the safety of the vectors which were used. Finally, carry out field trial experiments and studying the stability of the transgene expression in long term.

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