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Study of alfalfa somatic embryos for recombinant protein production

(Spine title: Recombinant protein production in alfalfa somatic embryos)

(Thesis format: Monograph)

by

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Graduate Program in Biology

A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science

The School of Graduate and Postdoctoral Studies

The University of Western Ontario

London, Ontario, Canada

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THE UNIVERSITY OF WESTERN ONTARIO
SCHOOL OF GRADUATE AND POSTDOCTORAL STUDIES

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Study of alfalfa somatic embryos for recombinant protein production

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ABSTRACT

Transgenic plants have been explored as a potentially inexpensive expression system for recombinant protein production. However, improvement in protein yield is still the most challenging problem limiting the commercial utilization of plant expression systems. In this study, alfalfa (*Medicago sativa* L.) somatic embryo was evaluated as a tissue for increased recombinant protein expression. Three heterologous genes: *β-glucuronidase* (*GUS*), *cholera toxin B subunit* (*CTB*), and *human interleukin 13* (*hIL-13*) were independently introduced into alfalfa via *Agrobacterium*-mediated transformation. Induced alfalfa somatic embryos store approximately two-fold more ectopically expressed proteins (as a percentage of total soluble protein) than vegetative organs such as roots, stems, and leaves. The foreign proteins CTB and hIL-13 could accumulate up to 0.15% and 0.18% of total soluble protein in alfalfa somatic embryos, respectively. These results indicate that alfalfa somatic embryos can serve as an efficient expression system to accumulate heterologous proteins.

Keywords: molecular farming, recombinant protein production, somatic embryos, alfalfa, β-glucuronidase, cholera toxin B subunit, human interleukin 13

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LIST OF ABBREVIATIONS

½ MSO	*Half-strength MSO*
2,4-D	2,4-Dichlorophenoxyacetic acid
4-MU	4-methylumbelliferone
ABA	Absisic acid
ABTS	2, 2'-Azinobis (3-ethylbenzothiazoline-6-sulfonic acid)-diammonium salt
ANOVA	Analysis of variance
ATP	Adenosine-5'-triphosphate
bp	Base pair
BSA	Bovine serum albumin
°C	*Degrees celsius*
CaMV	Cauliflower mosaic virus
cm	Centimeter
CT	Cholera toxin
CTB	Cholera toxin B subunit
DNA	Deoxyribonucleic acid
dNTPs	2'-deoxynucleoside 5'-triphosphates
ECL	Enhanced chemiluminescene
E. Coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
g	Gram

GFP	Green fluorescent protein
GUS	β-glucuronidase
hIL-13	Human interleukin 13
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase
HSPs	Heat shock proteins
IgG	Immunoglobulin G
kb	Kilo base pair
kDa	Kilo Dalton
l	Liter
LB	Luria-Bertani
LEA	Late embryogenesis abundant
LTB	Heat-labile toxin B subunit
M	Molar
mg	Milligram
ml	Millilitre
mM	Millimolar
MS	Mass Spectrometry
MUG	4-methylumbelliferyl-beta-D-glucuronide
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
ng	Nanogram
nm	Nanometre

nos	Nopaline synthase
NPT II	*Neomycin phosphotransferase II*
OD	Optical density
PBS	Phosphate-buffered saline
PBST	0.1% v/v Tween-20 in Phosphate-buffered saline
PCR	Polymerase chain reaction
PMSF	Phenylemethylsulphonyl fluoride
PVPP	Polyvinylpolypyrrolidone
rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
Ti	Tumor-inducing
TSP	Total soluble protein
µl	Microliter
µM	Micromolar
V	Volt

CHAPTER 1—INTRODUCTION

1.1 Plant growth and product accumulation

Plants are essential to the survival of our planet: to its ecology, biodiversity, and climate. They help to maintain human health by providing the basis for nutrition, shelter, clothing, and energy (McCormick and Tjian 2010). Most plants initiate their development from seeds, which are typically composed of seed coats, embryos, and endosperms. After germination, embryos will grow into mature plants, which are made up of three basic organs: roots, stems, and leaves. Leaves provide an essential structure for photosynthesis, the only process of converting the sun's radiant energy into organic chemical energy sources such as glucose, while roots and stems respectively absorb and transport different chemicals for plant growth. Seeds are formed from the reproductive shoot, or flower, completing the life cycle in seed plants (Campbell and Reece 2005). Somatic embryos, resulting from asexual reproduction, can act as an alternative mean of propagating plants (Namasivayam 2007).

The process of photosynthesis can be divided into two main parts: the light reactions and the carbon reactions. In the light reactions, light energy from the sun is absorbed and used to drive the generation of adenosine-5'-triphosphate (ATP) and reduced nicotinamide adenine dinucleotide phosphate (NADPH). The carbon reactions catalyzing the reduction of CO_2 to carbohydrates are coupled with the consumption of ATP and NADPH. The carbohydrates synthesized can serve multiple functions. They can be converted into organic compounds to maintain normal plant growth, or to be consumed by cellular respiration. Excess carbohydrates can be converted into storage

forms of energy or converted into secondary metabolites for plant defenses (Taiz and Zeiger 1998).

Although the carbohydrates are synthesized in the chloroplasts of plant leaves, seeds act as storage organs in many plant species. The synthesized carbohydrates accumulating in plant vegetative tissues during the day are transferred or utilized at night (Taiz and Zeiger 1998) to maintain spacious room for choloplast movement to regulate light absorption. In seeds, synthesized carbohydrates can be converted and deposited in large quantities as storage proteins, starches, and lipids, to be used for seed germination and early seedling growth. Seed storage bodies (Herman and Larkins 1999) and oleosomes (Harwood 1997) are formed during seed development to store proteins and triacylglycerols, respectively. The percentage of protein in legume seeds and cereal grains is 20-40% and 5-15% of dry weight, respectively, while protein in a typical leaf comprises 3-5% of its dry matter (Copeland and McDonald 2001). Thus, the storage capacity of seeds is several-fold higher than those of the vegetative tissues.

Embryogenesis is a developmental process in which a single-celled zygote transforms into a multicellular embryo. In dicotyledons, embryogenesis includes four distinct morphological stages: globular, heart, torpedo, and cotyledon stages. Globular stage is characterized by a radially symmetrical sphere of cells which is established from a precise pattern of initially synchronous cell divisions. Next, a bilaterally symmetrical heart stage embryo is produced by rapid cell division at two regions that ultimately lead to outgrowth of cotyledons. Then, the elongation of radicles, hypocotyls, and cotyledons produces torpedo stage embryos. Lastly, extensive growth of cotyledons results in the formation of cotyledon stage embryos, in which almost 90% of the total mass of the

embryos was stored in cotyledons (Taiz and Zeiger 1998). In dicotyledons, the cotyledons are the main storage organs of the seed. In contrast, most monocotyledons, such as corn (*Zea mays* L.) and wheat (*Triticum aestivum* L.), accumulate the majority of their nutrients in endosperms. Developmentally regulated dehydration leading to the loss of water ends seed development in most dicotyledons and monocotyledons (Copeland and McDonald 2001).

Legumes are typical dicotyledons which contain very high levels of storage proteins in cotyledons. Seed storage proteins of legumes have been classified into three major families: 2S albumins, 7S globulin, and 11S globulin (Shewry et al. 1995). In soybean (*Glycine max* L.) seeds, 7S and 11S storage proteins constitute appropriately 70% of the total seed protein at maturity and 30-40% of the mature seed weight (Meinke et al. 1981). In mature alfalfa (*Medicago sativa* L.) seeds, three major families, 11S medicagin, 7S alfin, and 2S albumins, comprise around 50% of the total extractable proteins (Krochko and Bewley 1988).

Besides primary products for normal plant growth and energy storage, plants produce a diverse array of organic compounds and several groups of stress-induced proteins against a variety of herbivores, pathogenic microbes, and stressful conditions. Many compounds serve as toxins against these intruders, while others function in absorbing harmful ultraviolet radiation or in reducing the growth of nearby competing plants. The heat shock proteins (HSPs), which are synthesized in response to higher than normal temperatures, help cells to better withstand heat stress. Late embryogenesis abundant (LEA) genes are considered to play a role in cellular membrane protection (Taiz and Zeiger 1998).

1.2 Molecular farming: its advantages and limitations

Recently, transgenic techniques have been used to exploit plants in the production of recombinant proteins for medicinal and industrial applications, frequently referred to as molecular farming (Ma et al. 2003). Molecular farming emerged when plant transformation techniques, established in the 1980s (Fraley et al. 1983), allowed for the insertion of a heterologous gene into a modified version of *Agrobacterium tumefaciens* tumor-inducing (Ti) plasmid, which is subsequently transferred into *Agrobacterium*. The transformed *Agrobacterium* can then be used to infect plant cells, allowing the integration of the foreign gene into the plant genome, followed by subsequent expression of this gene (Hooykaas and Schilperoort 1992). Higher plants like petunia (*Petunia hybrida*) and tobacco (*Nicotiana tabacum*) were first transformed using *Agrobacterium* (Fraley et al. 1983). The successful expression of a human growth hormone fusion protein in tobacco and sunflower (*Helianthus annuus* L.) callus tissue (Barta et al. 1986), a human interferon in turnip (*Brassica rapa* L.; De Zoeten et al. 1989), and a human serum albumin in tobacco and potato (*Solanum tuberosum* L.; Sijmons et al. 1990), demonstrated that it is possible to utilize plants as production systems for recombinant pharmaceuticals. The successful expression of functional antibodies in tobacco leaves indicated that sufficient protein post-translational modifications necessary for antibody activity also can take place in plants (Hiatt et al. 1989; Düring et al. 1990). The fact that the first proteins produced in plants, including antibodies and veterinary vaccines, are now approaching the market proves that plants can act as efficient bioreactors for large-scale recombinant protein production (Twyman et al. 2005).

Producing recombinant proteins in plants has many advantages. Without the requirement of fermenters and skilled workers, the cost of plant-based production systems, including capital and running costs, can be significantly lower than transgenic animal cell culture (Schillberg et al. 2003). Furthermore, compared with bacterial expression systems in which many complex recombinant proteins, antibodies in particular, are usually unable to be correctly folded, assembled, and modified (Swartz 2001), plant-based production systems can produce these proteins more accurately. This is because the protein-synthesis and post-translational pathways are highly conserved between plants and animals, which allows for more accurate heterologous bioreactive protein production (Ma et al. 2003). Moreover, as a production system for pharmaceutical proteins, plants are safer than animals because they generally lack human specific pathogens including viruses, prions, and oncogenic deoxyribonucleic acid (DNA) sequences (Ma et al. 2003).

Until now, several different strategies for recombinant protein production in plants have been utilized. Among them, nuclear transformation is the most frequently used method, by introducing heterologous DNA into the nuclear genome of a germ cell or a dedifferentiated cell by *Agrobacterium* infection. Therefore, the heterologous genes can be stably expressed under the control of promoters in the entire transgenic plant. Although it has been over 20 years since scientists first exploited this technique, there are many factors affecting the commercial use of this plant expression system. The most important factor is achieving adequate recombinant protein yields (Twyman et al. 2003). The seeds can accumulate foreign proteins up to 1% of total soluble protein (Lau and Sun 2009). The cumulative levels of recombinant proteins in vegetative tissues of transgenic

crops are even lower than those in the seeds of transgenic cereals or legumes (Daniell et al. 2001). This is probably because the vegetative tissues are not natural storage organs. Recombinant protein production in transgenic fruit is similarly lower than transgenic seeds (Daniell et al. 2001).

Besides nuclear transformation, several other strategies, such as chloroplast transformation, viral transfection, and plant cell culture, are also used for recombinant protein production in plants. Chloroplast transformation, introducing DNA into the chloroplast genome (Svab et al. 1990), has several advantages including a high transgene copy number and stable mRNA transcription, which can lead to very high levels of expression that, in extreme cases may exceed 25% of the total soluble protein (Tregoning et al. 2003). However, the major limitation of the chloroplast transgenic system is that, like bacterial expression systems, chloroplasts do not always carry out correct protein modification. Furthermore, technical challenges still exist for the use of chloroplast transformation in many plant species (Khan and Maliga 1999). Two other strategies including viral transfection and plant cell culture have their own limitations, including the ability for correct protein modification and low protein yields. Therefore, new expression systems are required to allow optimal protein specific expression and achieve adequate yields of recombinant proteins.

1.3 Somatic embryos: potential organs that may produce higher levels of recombinant proteins

Somatic embryos are usually induced from differentiated cells on media, through the process of dedifferentiation and somatic embryogenesis which is a unique phenomenon of higher plants (Namasivayam 2007). Steward et al. (1958) initially

reported somatic embryogenesis of carrots in tissue culture, and since then, it has been demonstrated in a large number of monocotyledons and dicotyledons (Ammirato 1983). The similarity in morphology and protein composition between somatic and zygotic embryos led us to hypothesize that the former could be used as efficient storage organs to accumulate heterologous proteins.

Like zygotic embryos, somatic embryos of dicotyledons also go through four developmental stages: globular, heart, torpedo and cotyledon stages. Cotyledons, radicles and hypocotyls outgrow after heart stage when large-scale production of protein occurs in the cotyledon (Dodeman et al. 1997). Protein composition in somatic embryos is similar to that of zygotic embryos. As seen in *Cyclamen persicum* Mill., the seed storage protein 11S globulin, identified by mass spectrometry (MS), was found to be expressed at a similar level in somatic embryos, zygotic embryos and the endosperm (Winkelmann et al. 2006). The composition of alfalfa storage proteins in somatic embryos is similar to that in zygotic embryos. In 14-day-old alfalfa somatic embryos, the amount of storage proteins is $< 20\%$ of that found in dry zygotic embryos (Krochko et al. 1994). LEA proteins, which accumulate to high levels in zygotic embryos, also exhibit high expression levels in carrot (*Daucus carota* L.) somatic embryos. Although all of the carrot *LEA* genes are found to be expressed in callus cells, most of the *LEA* transcripts increase significantly in quantity within somatic embryos at the heart stage (Wilde et al. 1988).

1.4 Alfalfa somatic embryogenesis and transformation

Alfalfa is a suitable candidate for recombinant protein production because of its favourable agronomic characteristics, routine somatic embryogenesis and gene transformation techniques. Widely grown as legume forage for cattle, alfalfa is known for

its high protein content and large amount of digestible fiber. As a legume, its ability to fix atmospheric nitrogen reduces the need for fertilization. Because it is harvested several times per year, it provides short-term, high levels of biomass. These characteristics provide an opportunity for low-cost, large-scale recombinant protein production (Busse et al. 2001; Khoudi et al. 1999). Both of alfalfa regeneration through somatic embryogenesis and gene transformation techniques, which are important for rapid amplification of these desired transgenic plants, are routine techniques right now. Maheswaran and Williams (1984) reported the first alfalfa regeneration through somatic embryogenesis. The successful genetic transformation of alfalfa, mediated by *Agrobacterium,* was also reported (Deak et al. 1986; Shahin et al. 1986; McKersie et al. 2000; Tesfaye et al. 2001).

The vegetative tissues of alfalfa have been used to express recombinant proteins in many labs, but no article reports using its somatic embryos for recombinant protein production yet. Fungal endochitinase 42 expressed in alfalfa exhibited lytic activity against glycol chitin, and inhibited germination of two fungal pathogens (Tesfaye et al. 2005). Recombinant C5-1 monoclonal antibody has been produced in transgenic alfalfa, and contained the common glycans of plants and mammals, which suggested this plant can produce recombinant Immunoglobulin G (IgG) having correct N-glycosylation (Bardor et al. 2003). The genes encoding the thermostable cellulases E2 and E3 were expressed under the control of the constitutive hybrid Mac promoter. Recombinant E2 was active and retained heat stability and this was an important first step in the development of alfalfa as a production system for cellulases (Ziegelhoffer et al. 1999).

To date, only Siberian ginseng (*Eleutherococcus senticosus*) mature somatic embryos (induced for three weeks) have been used to express the heat-labile toxin B subunit (LTB) of *Escherichia coil* (*E. coli*), which represented approximately 0.36% of the total soluble protein (Kang et al. 2006). However, the expression levels of recombinant proteins in somatic embryos have not been compared with those in vegetative tissues to test the benefits of this expression system.

1.5 β-glucuronidase, cholera toxin B subunit, and human interleukin-13

To test whether heterologous genes can be efficiently expressed in alfalfa somatic embryos, *β-glucuronidase* (*GUS*), *cholera toxin B subunit* (*CTB*), and *human interleukin-13* (*hIL-13*) genes were used in this study. The GUS protein, an enzyme from *E. Coli*, catalyzes the hydrolysis of a wide variety of β-glucuronides, many of which are available commercially as spectrophotometric, fluorometric and histochemical substrates. Jefferson et al (1987) first reported the ectopic expression of the *GUS* gene in plants, a commonly used marker for analysis of gene expression in transformed plants nowadays. There are many advantages for expression this marker in plants. Higher plants have no intrinsic glucuronidase activity, thus heterologous expressed GUS protein can be measured specifically. A very small amount of transformed plant tissue is enough for fluorometric assays to measure this accumulated heterologous protein accurately. Moreover, GUS protein is stable and tissue extracts continue to have high levels of activity after prolonged storage (Jefferson et al. 1987).

CTB, an 11.6-kDa non-toxic B subunit of cholera toxin (CT) produced by the bacterium *Vibrio cholerae*, is a candidate oral vaccine for cholera, an acutely dehydrating, watery diarrhoeal disease caused by cholera colonization of the small

intestine (Holmgren et al. 1993). The non-toxic B subunits mediate binding to receptors on the eukaryotic cell surface and provide a safe way for the evaluation of these toxins as mucosal adjuvant in humans and animals (McGhee et al. 1992). The expression of recombinant CTB antigen in plants offers several advantages, including low production cost, ease of administration (ingestion of plant material) and good storage properties. This protein had been expressed at a level of 0.3% of total soluble protein in potato (Arakawa et al. 1997), 0.095% in tobacco (Wang et al. 2001), 0.04% in the fruits of tomato (*Lycopersicon esculentum* Mill.; Jani et al. 2002), 0.48% in the root of transgenic carrot (Kim et al. 2009), and 0.24% in lettuce (*Lactuca sativa* L.; Kim et al. 2006).

hIL-13, a secreted T-cell-derived cytokine, plays an important role in the regulation of inflammatory and immune responses in human beings (Minty et al. 1993). There are two forms for hIL-13 *in vivo*, unglycosylated and glycosylated, which are inactive and active forms, respectively (Cannon-Carlson et al. 1998). hIL-13 has been reported as a potential therapeutic agent for the treatment of cancers (Shimamura et al. 2006), inhibition of human immunodeficiency virus (HIV) replication *in vitro* (Montaner et al. 1993), and for host protection against gastrointestinal nematode infection (Bancroft et al. 1998). However, only small-scale production of hIL-13 could be achieved in cell culture using mouse myeloma cells (Cannon-Carlson et al. 1998) or *E. coli* (Thompson and Debinski 1999). Compared with murine cell-derived hIL-13, which exists in disulphide-linked oligomeric forms and has no biological activity (Cannon-Carlson et al. 1998), recombinant hIL-13 expressed in tobacco exhibited multiple biologically active molecular forms, each associated with different N-linked glycosylations (Wang et al. 2008).

1.6 Objective

The aim of this study was to evaluate the ability of alfalfa somatic embryos to accumulate recombinant proteins. Three foreign genes *GUS*, *CTB*, and *hIL-13* were introduced into alfalfa to test the efficiency of recombinant protein accumulation in somatic embryos derived from this plant. The accumulation levels of these three recombinant proteins in somatic embryos were compared with their accumulation in vegetative organs. This study may lead to the development of an efficient system for commercially relevant protein production in plants.

CHAPTER 2—MATERIALS AND METHODS

2.1 Plant materials

Alfalfa clone N442 (developed by Stephen R Bowley) with high ability of somatic embryo production in tissue culture was cultivated in 10-cm Magenta containers with half-strength MSO (½ MSO) solid medium (Table 1) in a tissue culture room at 25°C with 16 h light/ 8 h dark.

2.2 Plant expression vectors

pCAMBIA 2301 binary vector (Cambia, Canberra, Australia) contains *GUS* cDNA driven by a CaMV 35S promoter (Figure 1; Odell et al. 1985). The pBI-CTB vector (provided by Dr. Shengwu Ma), derived from the pBI 121 vector (Jefferson et al. 1987), contains a dual-enhancer CaMV 35S promoter (Kay et al. 1987) followed by the *CTB* cDNA (Figure 2A). The pCAM hIL-13-GFP vector, which was constructed in this study, contains *hIL-13* cDNA fused with *green fluorescent protein* (*GFP*; Shimomura et al. 1962) cDNA driven by a dual-enhancer CaMV 35S promoter (Figure 2B). All three vectors express *neomycin phosphotransferase II* (*NPT II*) to confer kanamycin resistantance.

2.2.1 Gene amplification and the introduction of flanking enzyme restriction sites

For construction of the pCAM hIL-13-GFP vector, the *hIL-13* and *GFP-SEKDEL* cDNAs were firstly cloned separately. The *hIL-13* cDNA was cloned by polymerase chain reaction (PCR) using primers 1 and 2 (Table 2), an intermediate vector (supplied by Dr. Shengwu Ma; Wang et al. 2008) as template. The *GFP* cDNA was cloned from pCAMBIA 1302 (Cambia, Canberra, Australia) by PCR using primers 3 and 4 (Table 2).

Table 1. The composition and functions of major media used for alfalfa transformation.

Medium	Basal medium	Growth regulator	Additional chemicals	Function
SH2K	Schenk and Hildebrandt (1972)	4.5 µM 2, 4-D, 0.9 µM kinetin	25mM proline, 0.4 mM thioproline, 50 mM potassium sulphate, 200 mg l^{-1}*myo*-inositol 2% (w/v) sucrose 0.25% (w/v) gelrite	Callus induction
BOi2Y	Blaydes (1966)	N/A	0.2% yeast extract, 100 mg l^{-1} *myo*-inositol 3% (w/v) sucrose 0.25% (w/v) gelrite	Embryo development
MSO	Murashige and Skoog (1962)	N/A	1 mg l^{-1} glycine 2% (m/v) sucrose 0.8% (w/v) agar	Embryo germination and plant growth

Note: The pH of all the media was adjusted to 5.7. ½ MSO solid medium contained the same amount of sucrose, glycine and agar concentration as MSO solid medium, but half the amount of MS components. 2,4-D, 2,4-dichlorophenoxyacetic acid. N/A, not available.

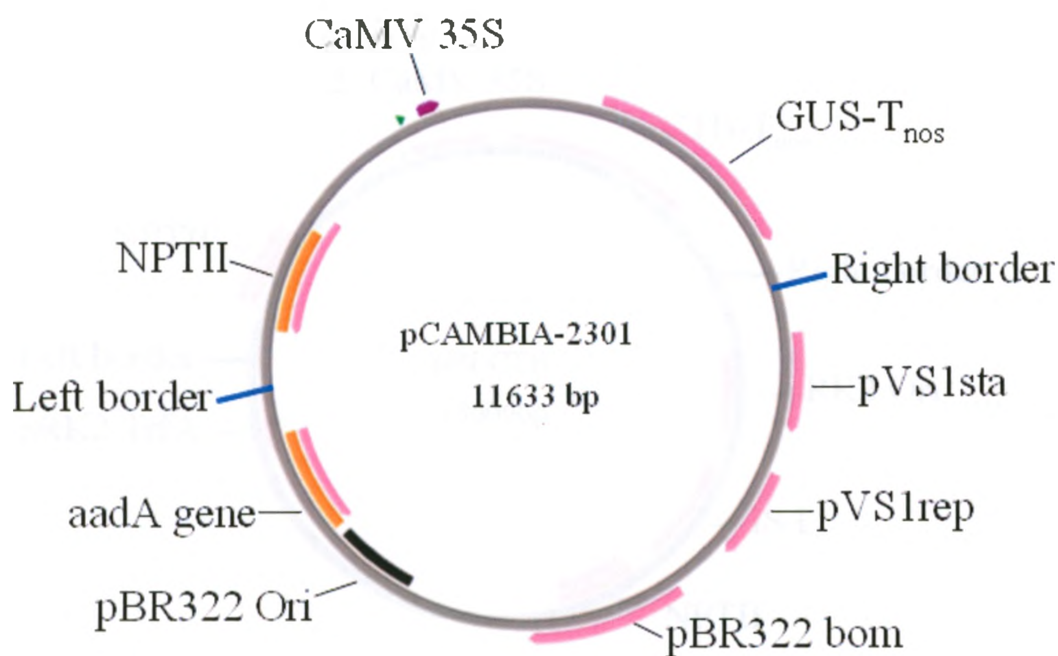
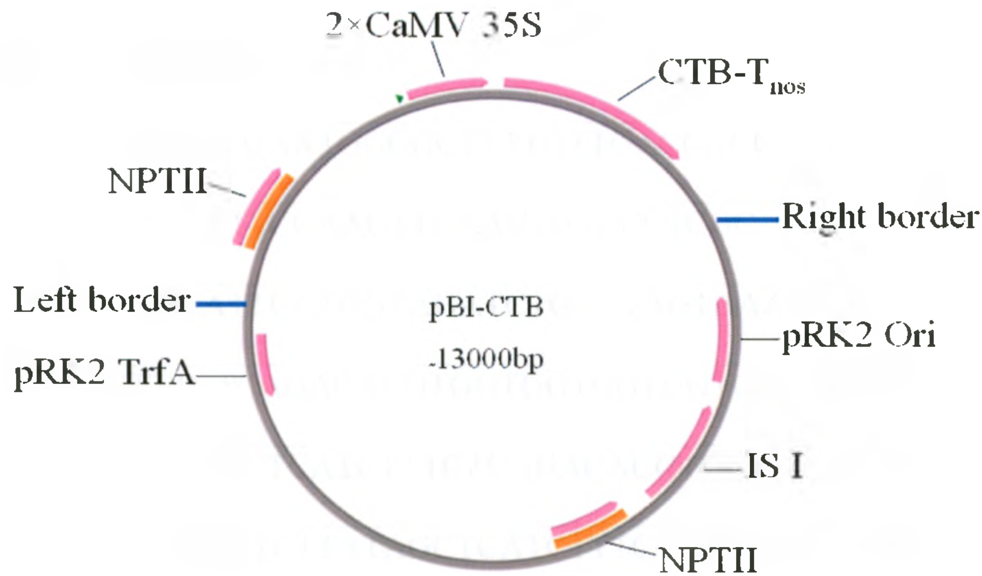


Figure 1. Map of the pCAMBIA 2301 vector. The sequence of pCAMBIA 2301 (AF234316) was obtained from National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). Online PlasMapper 2.0 Software (http://wishart.biology.ualberta.ca/PlasMapper/index.html) was used to draw pCAMBIA 2301 vector map. Both *neomycin phosphotransferase II* (*NPT II*) and *aadA* confer kanamycin resistantance. The *GUS* cDNA is driven by the CaMV 35S promoter.

A



B

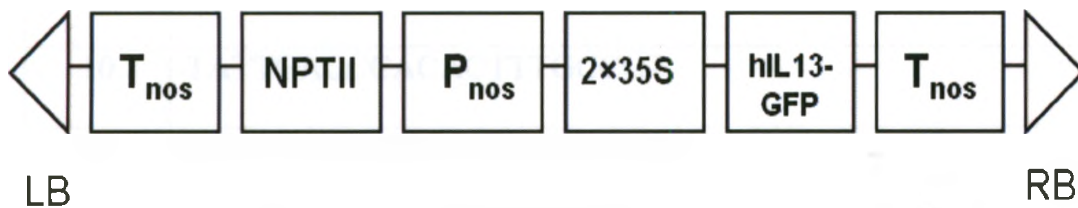


Figure 2. Map of the pBI-CTB expression vector and T-DNA region of the pCAM hIL-13-GFP vector.

(A) Map of the pBI-CTB vector. The pBI-CTB vector was derived from the pBI 121 vector (HM047294), the sequence of which was obtained from National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). Online PlasMapper 2.0 Software (http://wishart.biology.ualberta.ca/PlasMapper/index.html) was used to draw pBI-CTB vector map. (B) The T-DNA region of the pCAM hIL-13-GFP vector. The non-T-DNA region of pCAM hIL-13-GFP vector was the same as that of pBI-CTB. 2×35S, a dual-enhancer cauliflower mosaic virus (CaMV) 35S promoter; LB, left border; RB, right border; Tnos and Pnos, terminal and promoter of nopaline synthase (nos); NPT II, *neomycin phosphotransferase II.*

Table 2. Primers used for PCR amplification of sequences required for pCAM hIL-13-GFP vector construction and transgenic plant selection.

Number	Primer sequence 5'-3'
1	GCTCTAGAATGGCGCTTTTGTTGACCACG
2	CGGAATTCCAAGTTGAACTGTCCCTCGC
3	CGGAATTCATGGTAGATCTGACTAGTAAAGGAG
4	CTTTCTCAGACACGTGGTGGTGGTGGTGGTGGCTAG
5	TTATAGCTCATCTTTCTCAGACACGTGGTGGTGGTG
6	GCGAGCTCTTATAGCTCATCTTTCTCAGACACGTGG
7	GAGGCTATTCGGCTATGACTG
8	ATCGGGAGCGGCGATACCGTA
9	CGTCCTGTAGAAACCCCAAC
10	TATTGACCCACACTTTGCCGT
11	TGCAGAATACCACAACACACAA
12	GCAATCCTCAGGGTATCCTTC

Note: All oligonucleotides were synthesized by Operon (Eurofins MWG Operon, AL, USA).

An endoplasmic reticulum retrieval signal (SEKDEL) (Munro and Pelham 1987) was added to the C- terminal of *GFP* by two round extension PCR using primers 3 and 5, and primers 3 and 6 (Table 2), separately. All PCR amplifications were conducted using an Eppendorf Mastercycler gradient thermocycler (Eppendorf, Hamburg, Germany). All primers contained flanking enzyme restriction sites for subcloning. *Xba*I and *Eco*RI restriction sites were incorporated at the 5'- and 3'- end of *hIL-13*; and *Eco*RI and *Sac*I restriction sites for *GFP-SEKDEL*. PCR reactions (50 μl) were set up with 50 ng of DNA template, two units of DNA polymerase (GenScript, Piscataway, NJ, United States; E00007), 1×PCR buffer, 200 μM 2'-deoxynucleoside 5'-triphosphates (dNTPs; GenScript; C01689) and 2 μM of each primer. All PCR reactions were conducted using a 56°C annealing temperature for 35 cycles except when amplifying *GFP-SEKDEL*, where a 65°C annealing temperature was used with the same number of cycles. The PCR products were analyzed by agarose gel electrophoresis and amplified DNA fragments with the correct size were gel extracted using a DNA gel extraction kit (Qiagen, Mississauga, ON, Canada).

2.2.2 pCAM hIL-13-GFP expression vector construction

The pCAMter X vector (provided by Laurian Robert) was used for the generation of pCAM hIL-13-GFP expression vector. The pCAMter X vector (~ 11 kb), derived from the pBIN19 vector (Bevan 1984), was modified to include a dual-enhancer CaMV 35S promoter. The PCR products for the pCAM hIL-13-GFP vector construct were subcloned into a TA cloning vector using T4 ligase (Invitrogen, Carlsbad, CA, United States; AM2141). The resulting plasmids were transformed into *E. coli* strain DH5α (Invitrogen) using the Gene Pulser II Electroporation System (Bio-Rad, Hercules, CA, United States).

The *E. coli* cells being cultured in selective Luria-Bertani (LB) liquid medium [1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) sodium chloride, pH 7.0] overnight were used for plasmid DNA isolation using a plasmid purification kit (Qiagen). The TA vectors carrying the *GFP-SEKDEL* cDNA were digested with *Eco*RI and *Sac*I; while the *hIL-13* carrying plasmid was digested with *Xba*I and *Eco*RI. The pCAMter X vector was first digested with *Eco*RI and *Sac*I. After gel purification using a DNA gel extraction kit (Qiagen), the linearized pCAMter X vector was ligated with the digested *GFP-SEKDEL* insert using T4 ligase. After another round of *E. coli* transformation and plasmid extraction, the vector of pCAMter-GFP was digested with *Xba*I and *Eco*RI, and ligated with the *hIL-13* cDNA. An *Eco*RI restriction site was added between *hIL-13* and *GFP* to create a two-amino-acid linker (glutamic acid-phenyl alanine). After a final round of *E. coli* transformation and plasmid extraction, the expression vector was confirmed by PCR amplification and sequencing.

2.3 *Agrobacterium* transformation

Purified plant expression vectors were transformed into the *Agrobacterium* strain GV3101 (Du et al. 1994) via electroporation which was subsequently used for alfalfa transformation. Transformed cells were allowed to recover in LB liquid medium by shaking horizontally at 28°C for 1 h and then grown on a LB solid medium with antibiotics kanamycin, rifampicin and gentamicin for about 48 h.

2.4 *Agrobacterium*-mediated alfalfa transformation and somatic embryogenesis

The procedure of alfalfa transformation and somatic embryo development (Du et al. 1994) is shown in Figure 3. Young petioles of wild type alfalfa were cut into ~ 1 cm fragments and pre-cultured on fresh SH2K solid medium (Table 1) for 48 h prior to

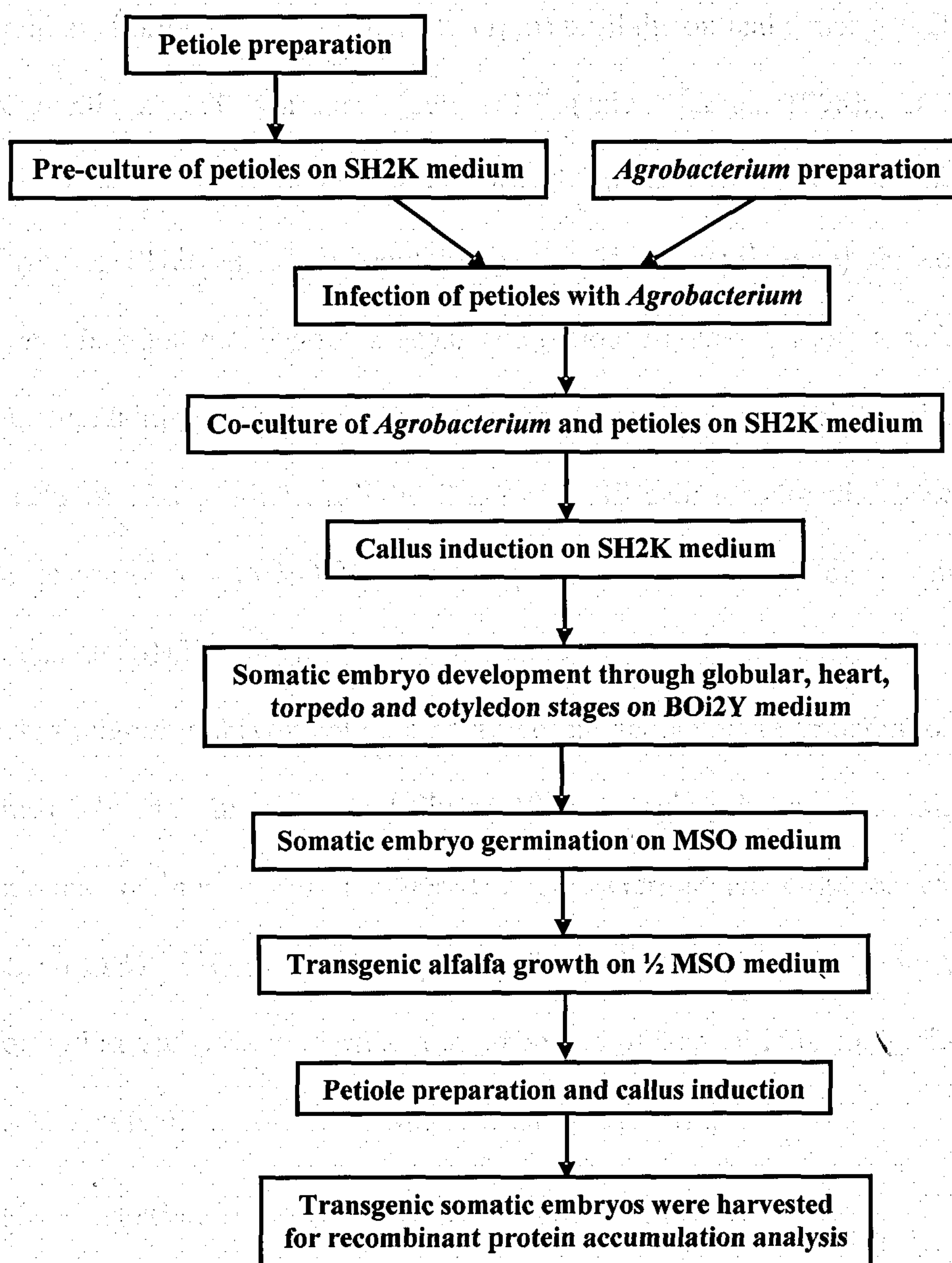


Figure 3. Procedure for alfalfa transformation and transgenic somatic embryo development.

transformation. *Agrobacterium* cultures carrying the expression vectors were grown in LB liquid medium in a 28°C shaker (200 rpm) until the optical density (OD) values of the culture media at 600 nm had reached 1.0 (Jaiwal et al. 1998). After infected by *Agrobacterium* in LB liquid medium for 5 minutes, these petiole fragments were transferred to SH2K medium (containing 20 μM acetosyringone) for co-culturing the *Agrobacterium* and petioles for 2 days. The petiole fragments were transferred to SH2K medium (containing 300 mg l^{-1} timentin and 75 mg l^{-1} kanamycin) and maintained for five weeks at 25°C with 16 h light/ 8 h dark until calli developed. These embryogenic calli were transferred to BOi2Y medium (Table 1; Blaydes 1996) containing the same antibiotics as SH2K medium and maintained at the same conditions for 2 weeks. Elongated mature embryos with well-formed cotyledons were transferred to MSO solid medium (Table 1; Murashige and Skoog 1962) carrying the same antibiotics as SH2K medium and maintained for another 2 weeks. Germinated embryos with roots were transferred to ½ MSO medium carrying 300 mg l^{-1} timentin in Magenta containers. All transformation steps were carried out under sterile conditions in a biological safety cabinet (Labconco, Kansas, MO, United States; Type A2).

2.5 DNA extraction and transgenic plant confirmation

2.5.1 DNA extraction

A basic iso-propanol method was used to extract genomic DNA from alfalfa transgenic lines (McKersie et al. 2000). Putative transgenic leaf tissues (0.1 g, around three leaves per line) were homogenized using a Tissue Mill (Retsch, Haan, Germany) for 1 minute. Four hundred μl of DNA extraction buffer [200 mM Tris-HCl, pH 7.5, 250 mM Sodium chloride, 25 mM ethylenediaminetetraacetic acid (EDTA), 0.5% (w/v)

Sodium dodecyl sulphate (SDS)] was added into 2 ml microcentrifuge tubes containing samples. Samples were left at room temperature for 1 hour and then centrifuged at 13000 rpm for 10 minutes (also at room temperature). Three hundred μl of supernatant was transferred into a new microcentrifuge tube and 300 μl of iso-propanol was added. After mixing at room temperature for 2 minutes, the DNA solution was centrifuged at 13000 rpm for 10 minutes. After the supernatant was discarded, the remaining DNA pellet was washed in 70% ethanol, centrifuged, dried in a vacuum, and then dissolved in 100 μl sterile distilled water. In total, the DNA of 12 putative alfalfa transgenic lines carrying *GUS*, 41 lines carrying *CTB*, and 15 lines carrying *hIL-13* were extracted for three independent times.

2.5.2 Multiplex PCR for confirming transgenic plants

Multiplex PCR, a variant of PCR which rapidly detects multiple sequences in the same reaction (Chamberlain et al. 1988), was performed using the same reaction setting as in **2.2.1**, but with multiple specific primers. The specific primers of *neomycin phosphotransferase II* (*NPT II*) gene, which confers resistance to kanamycin, and three transgenes was used for screening putative transgenic plants. Primers are shown in Table 2: *NPT II*, primers 7 and 8; *GUS*, primers 9 and 10; *CTB*, primers 11 and 12; *hIL-13*, primers 1 and 2.

2.6 Transgenic somatic embryo induction

Transgenic somatic embryos for recombinant protein accumulation analysis were induced using general *Agrobacterium*-mediated alfalfa transformation steps. Young petioles of transformed alfalfa were cut into small fragments and transferred to SH2K medium for callus induction, and then transferred to BOi2Y medium for transgenic

somatic embryo development. After calli were maintained on BOi2Y medium for two weeks, the somatic embryos that displayed well-formed cotyledons were harvested. All somatic embryogenesis steps were carried out under sterile conditions.

2.7 GUS histochemical staining

The protocols of GUS staining were taken from Jefferson et al (1987). GUS staining solution {0.1 M NaH_2PO_4, pH 7.0, 0.5 mM $K_3[Fe(CN)_6]$, 0.5 mM $K_4Fe(CN)_6.3H_2O$, 10 mM EDTA, 1% (v/v) Triton X-100} was added to 2-ml microcentrifuge tubes containing two-month-old transgenic vegetative tissues and two-week-old transgenic somatic embryos. These tubes were placed in a vacuum for 10 minutes to infiltrate the staining solution into plant tissues and then were incubated at 37°C overnight. Seventy percent ethanol was then used to decolour chlorophylls.

2.8 Quantitative GUS assay

2.8.1 Total soluble protein extraction for GUS assay

The protocols of total soluble protein extraction and following quantitative GUS assay were taken from Jefferson et al (1987). Two-month-old alfalfa transgenic vegetative tissues and two-week-old somatic embryos were ground under liquid nitrogen with a mortar and pestle. The mixture of two-month-old alfalfa wild type vegetative tissues and two-week-old somatic embryos was used as negative controls. GUS extraction buffer (50 mM Na_3PO_4, pH 7.0, 10 mM β-mercaptoethanol, 10 mM EDTA, pH 8.0, 0.1% (v/v) Triton X-100) was added to 2-ml microcentrifuge tubes (3 μl /mg). Samples were homogenized using a vortex and centrifuged at 13000 rpm for ten minutes

at 4 °C. The supernatant was re-centrifuged at the same speed until it was not cloudy. The supernatant represented the GUS solution.

2.8.2 Protein determination by Bradford assay

Undiluted Bradford reagent Coomassie G-250 Dye (Sigma-Aldrich, St. Louis, MO, United States; B5133) was added to a 96 well plate (200 µl per well) and 3 µl of undiluted samples were added per well. The plate was read at 595 nm using a microplate reader (Bio-Rad, 550) following a five-minute room-temperature incubation. The concentrations of sample proteins were determined based on a standard curve obtained by plotting the measured absorbance of the protein-dye conjugates versus the known concentration of bovine serum albumin standard (BSA; Bradford 1976).

2.8.3 GUS protein quantification

GUS activity was measured by monitoring cleavage of the β-glucuronidase substrate 4-methylumbelliferyl β-D-glucuronide (MUG) to 4-methylumbelliferone (4-MU; Jefferson et al., 1987). Two ml of MUG incubation buffer (1 mM MUG in GUS extraction buffer) was added to a 2-ml microcentrifuge tube and pre-incubated at 37°C for 5 minutes. Two hundred µl of GUS solution from different organs was added and mixed. Then 3×100 µl of reaction mixture (three replicates) was transferred into 3 separate 2-ml microcentrifuge tubes containing 1.9 ml stop buffer (0.2 M Na_2CO_3) on ice. The released 4-MU was quantified immediately in a recording fluorometer (Shimadzu, Kyoto, Japan; RF-Mini 150) at 595 nm. The OD values of samples were used to compare that of known concentration of 4-MU in stop buffer. The assay was performed three times using three independent plant materials. Each solution from different organs was

conducted with three replicates. Two-way analysis of variance (ANOVA) and Turkey test were run using SigmaPlot v11.0 to determine the variance of GUS activity in somatic embryos compared with those in vegetative organs within each transgenic lines, and the variance of protein accumulation in each organ between transgenic lines.

2.9 Western blotting for characterizing CTB and hIL-13

2.9.1 Total soluble protein extraction

Two-month-old alfalfa transgenic vegetative tissues and two-week-old somatic embryos were ground under liquid nitrogen. The mixture of two-month-old alfalfa wild type vegetative tissues and two-week-old somatic embryos was used as negative controls. One hundred and fifty µl of protein extraction buffer {2% (w/v) polyvinylpolypyrrolidone (PVPP; Sigma-Aldrich; P6755), 0.0001% (w/v) leupeptin (Invitrogen), 1 mM EDTA, 1 mM phenylemethylsulphonyl fluoride (PMSF; Sigma-Aldrich; P7626), 100 mM Ascorbic Acid (Sigma-Aldrich; A4403) in phosphate-buffered saline (PBS)-Tween 20 [PBST; 0.1% (v/v) Tween-20 in PBS]} was added to each 1.5-ml microcentrifuge tube containing 100 mg of plant tissues. Samples were homogenized using a vortex and then centrifuged for 15 minutes at 14000 rpm and 4°C. The supernatant which contains the extracted total soluble proteins was analyzed using a Bradford assay to detect the concentrations of different protein samples (Conley et al. 2009).

2.9.2 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was used to determine the same amounts of loaded proteins. Thirty five µg total soluble protein samples from each transgenic roots, stems, leaves, somatic

embryos, and wild type alfalfa were resolved on a 12% SDS gel (Mini-gel apparatus, Bio-Rad). Prestained protein marker (New England Biolabs; P7708) was loaded to show the molecular weights of proteins. Electrophoresis was performed at 100V for two to three hours until the dye front was 1 cm from the bottom of the plate. After electrophoresis, gels were stained by Coomassie blue G-250 (MP Biomedicals, Solon, Ohio, USA; R25068) and then destained in 10% acetic acid to visualize total soluble protein. Positive controls for Western blotting were not loaded here because their amounts were so small that they were not stained clearly in the gels.

2.9.3 Western blotting

After using SDS-PAGE to confirm the amount of proteins of each sample, the same amounts of proteins were loaded again with positive controls: bacterial derived CTB (Sigma-Aldrich, C-1655) for probing CTB or Positope, a 53 kDa recombinant protein specifically engineered to contain GFP (Invitrogen, R900-50) for probing hIL-13, respectively. The same prestained protein marker as above was loaded to show the molecular weights of proteins. After gel electrophoresis, proteins in the gel were electrotransferred onto a PVDF membrane (Bio-Rad, 162-0184) using a semi-dry electroblotter (Bio-Rad). The membranes were immersed with blocking buffer [1% (w/v) BSA in PBST] overnight at 4°C. After 3×10 minute washes with PBST buffer, the membranes were probed with rabbit anti-cholera toxin antibody (Sigma-Aldrich, C-3062) or anti-GFP antibody (Sigma-Aldrich, G1544) at a dilution of 1:10000 (v/v) in the blocking buffer (at room temperature for one hour). After 3×10 minute washes with PBST buffer, the membranes were incubated with horseradish peroxidase (HRP) conjugated anti-rabbit IgG (Sigma-Aldrich, A0545) as the secondary antibody at a

dilution of 1:10000 (v/v). The signal was detected by enhanced chemiluminescene (ECL; Amersham Pharmacia, Uppsala, Sweden). The bands of prestained protein marker on PVDF membranes were used to estimate the molecular weights of proteins probed.

2.10 Quantification of CTB and hIL-13 protein levels in transgenic alfalfa plants using enzyme-linked immunosorbent assay (ELISA)

Following protein extraction and quantification by the Bradford Assay, the relative amounts of alfalfa-accumulated CTB and hIL-13 proteins were quantified using an indirect ELISA compared to known amounts of bacterial derived CTB and Positope. Briefly, 100 μg total protein samples from transgenic and wild type alfalfa, and a series of gradient positive controls (0 ng-125 ng in protein extraction buffer) were aliquotted to the wells of a 96-well microtiter plate (Agdia, Elkhart, IN, United States) overnight at 4 °C. Non-specific binding was blocked with 200 μL PBST containing 1% (w/v) BSA for 2 hours at room tempreture. After three washes with 200 μL PBST, the plate was incubated with rabbit anti-CTB antibody (Sigma-Aldrich, C-3062) or anti-GFP antibody (Sigma-Aldrich, G1544) at a dilution of 1:8000 (v/v) in PBST for 2 hours at room temperature. After washing with 200 μL PBST, the plate was incubated with HRP conjugated anti-rabbit IgG (Sigma-Aldrich, A0545) as the secondary antibody at a dilution of 1:10000 (v/v) in PBST for another 2 hours at room temperature. After washing with PBST, the plate was developed with 2,2'-Azinobis (3-ethylbenzothiazoline-6-sulfonic acid)-diammonium salt (ABTS) substrate solution [0.1 M anhydrous citric acid, 0.03% (w/v) ABTS, pH 4.35, 0.03% (v/v) H_2O_2, which was added immediately before use]. The OD was measured at 405 nm in a microplate reader (Bio-Rad, 550; Li et al. 2006). The percentage of plant TSP was calculated by reference to ELISA standard curves

constructed with the positive control proteins. Two-way ANOVA and Turkey test were run using SigmaPlot v11.0 to determine the variance of recombinant protein concentrations in somatic embryos compared with those in vegetative organs within each transgenic line, and variance of concentrations in each organ between transgenic lines.

CHAPTER 3—RESULTS

3.1 Construction of plant expression vector pCAM hIL-13-GFP

To construct the plant expression vector pCAM hIL-13-GFP, the cDNAs of *hIL-13* and *GFP* were amplified by PCR individually, and analyzed by agarose gel electrophoresis (Figure 4). After confirmation by DNA sequencing, constructed pCAM hIL-13-GFP (Figure 4) was used for alfalfa transformation.

3.2 Transgenic plants production

3.2.1 *Agrobacterium*-mediated transformation

Cultured wild type alfalfa on ½ MSO medium was shown in Figure 5A. Petiole fragments from wild type alfalfa were pre-cultured on SH2K medium (Figure 5B). Calli developed from infected petioles grew on the same medium (Figures 5C and 6A). Small green somatic embryos were observed when calli were moved to BOi2Y medium for four days (Figures 5D and 6B). Germinated somatic embryos displayed an elongated root as well as two outgrown cotyledons (Figure 5E and 6C). Putative transformed alfalfa plants were obtained and grown in ½ MSO medium (Figure 5F). In total, three independent transformations using *Agrobacterium* strains carrying the binary vectors: pCAMBIA 2301, pBI-CTB, and pCAM hIL-13-GFP were conducted, respectively.

3.2.2 Confirmation of transgenic plants

Multiple PCR was used to identify transgenic plants. In total, 12 out of 12 putative transformed plants carrying the *GUS* were obtained and identified using *NPT II* and *GUS* specific primers (Figure 7). Thirty-eight out of 41 putative transformed plants carrying

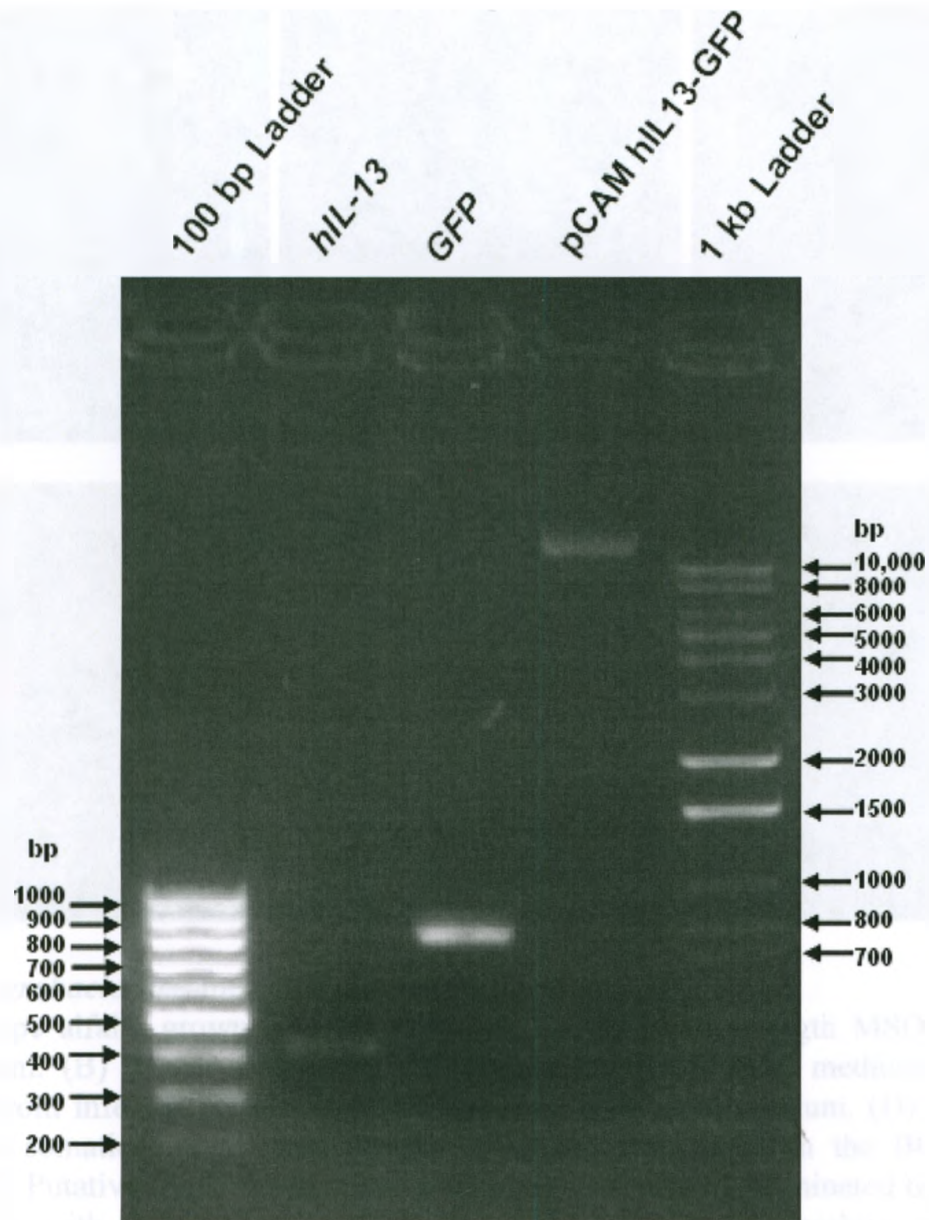


Figure 4. Agarose gel electrophoresis of PCR amplification products, *hIL-13* and *GFP* cDNAs for pCAM hIL-13-GFP vector construction, and constructed pCAM hIL-13-GFP vector.

The first and last lanes are the 100 bp ladder and 1kb ladder, respectively. The middle three lanes are *hIL-13*, *GFP* and pCAM hIL-13-GFP vector. Fragment sizes were as expected, 415 bp, 790 bp and around 12 kb, respectively.

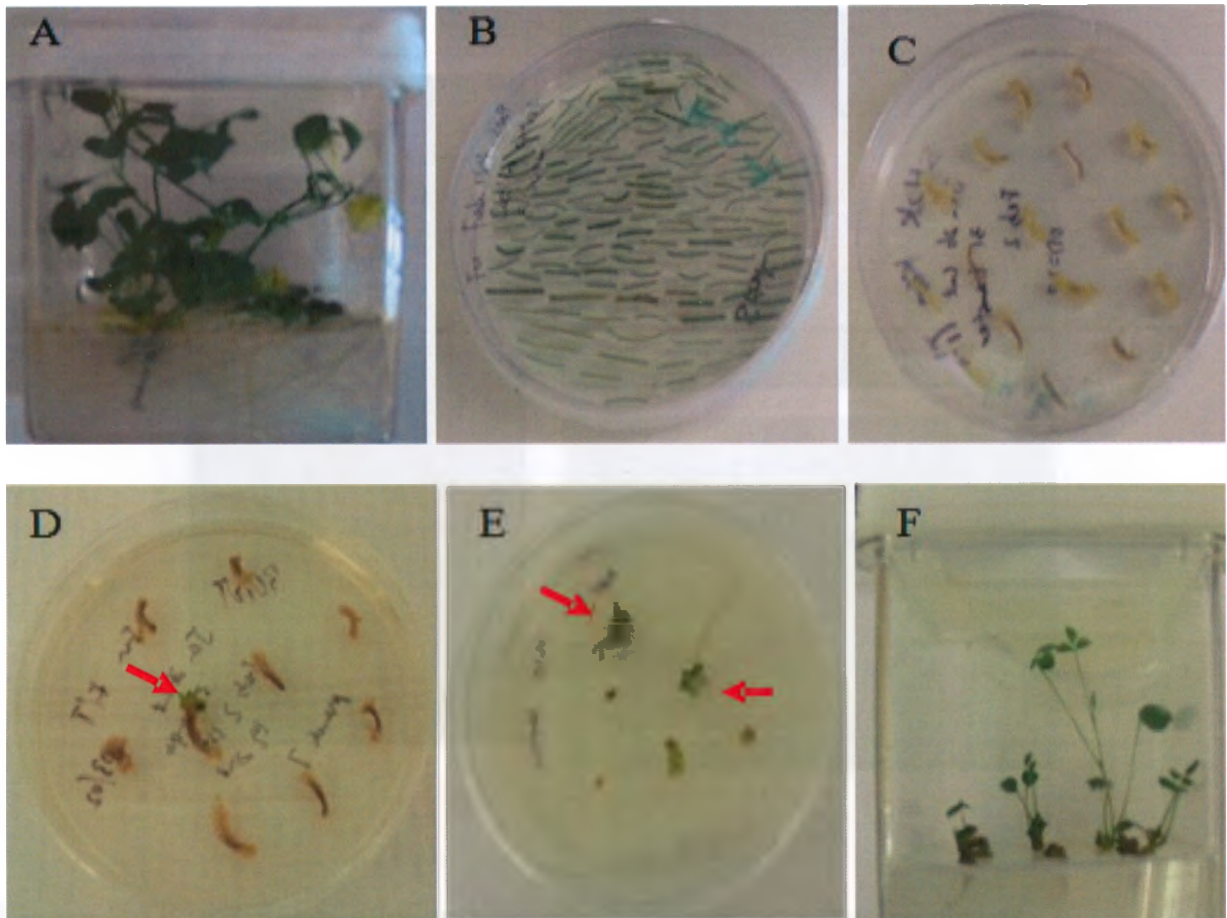


Figure 5. *Agrobacterium*-mediated alfalfa transformation.
(A) Wild type alfalfa grown in a Maganta box carrying half-strength MSO (½ MSO) solid medium. (B) Petiole fragments pre-cultured on SH2K solid medium. (C) Calli developed from infected petiole fragments on the SH2K solid medium. (D) A putative transformed somatic embryo (red arrow) developed from calli on the BOi2Y solid medium. (E) Putative transformed somatic embryos (red arrow) germinated on the MSO solid medium with elongated roots. (F) Putative transgenic somatic embryos grew on ½ MSO solid medium.

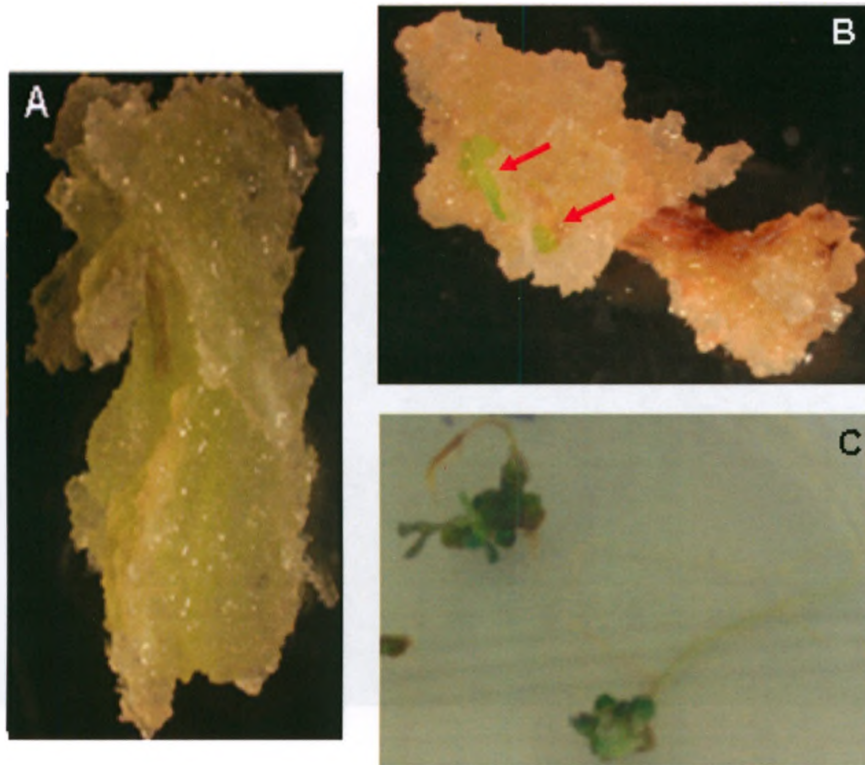


Figure 6. The development of putative alfalfa transgenic somatic embryos.
(A) A callus developed on SH2K medium. (B) Putative transformed somatic embryos grown on BOi2Y medium (red arrow). (C) The putative transformed somatic embryos germinated and exhibited an elongated root on MSO medium.

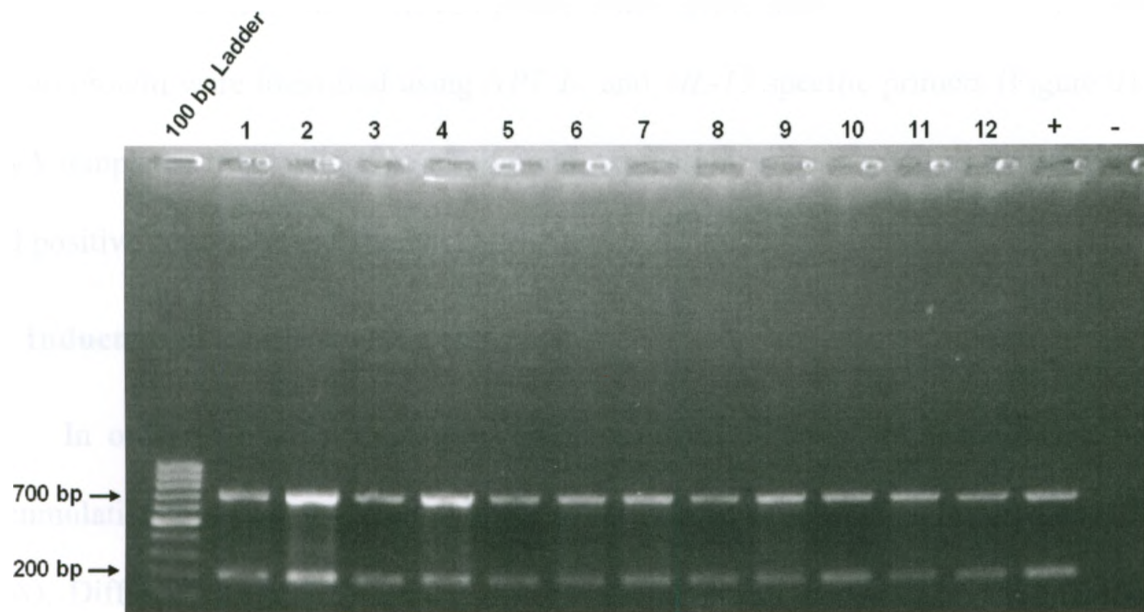


Figure 7. Confirmation of alfalfa carrying *GUS* by multiplex PCR.
Neomycin phosphotransferase II (*NPT II*) and *β-glucuronidase* (*GUS*) specific primers, generating 700 bp and 198 bp fragments, respectively, were used. Lane1 to 12, PCR products with DNA template from independent transgenic line 1 to 12; + and -, positive control using pCAMBIA 2301 vector as a PCR template and negative control without PCR template, respectively. The 100 bp DNA ladder is indicated.

CTB were screened out using *NPT II* and *CTB* specific primers (Partial results are shown in Figure 8). Fourteen out of fifteen putative transgenic plants carrying the *hIL-13-GFP* fusion protein were identified using *NPT II*, and *hIL-13* specific primers (Figure 9). The DNA templates from wild type alfalfa and expression vectors acted as negative control and positive control, respectively.

3.3 Induction of alfalfa somatic embryos

In order to harvest transgenic alfalfa somatic embryos for recombinant protein accumulation analysis, somatic embryos were induced on BOi2Y solid medium (Figure 10A). Different stages of somatic embryos development (globular, heart, torpedo and cotyledon stages) are shown in Figure 10B.

3.4 Analysis of recombinant protein accumulation in transgenic alfalfa

3.4.1 GUS protein detection

As expected, all organs of GUS transgenic lines exhibited blue color, indicating that active GUS enzyme had been produced in transgenic plants. GUS staining in organs of transgenic line 5, which exhibit the deepest blue color, was shown in Figure 11. GUS protein accumulated in somatic embryos of all development stages (Figure 11D), being mainly localized in the cotyledons.

3.4.2 GUS protein quantification

Quantitative GUS assay was used to quantify this protein accumulation in different plant organs of three random independent GUS transgenic lines, and wild type alfalfa was also included as a negative control. Based on analysis of two-way ANOVA, there were significant variances among GUS protein accumulation levels within each transgenic line,

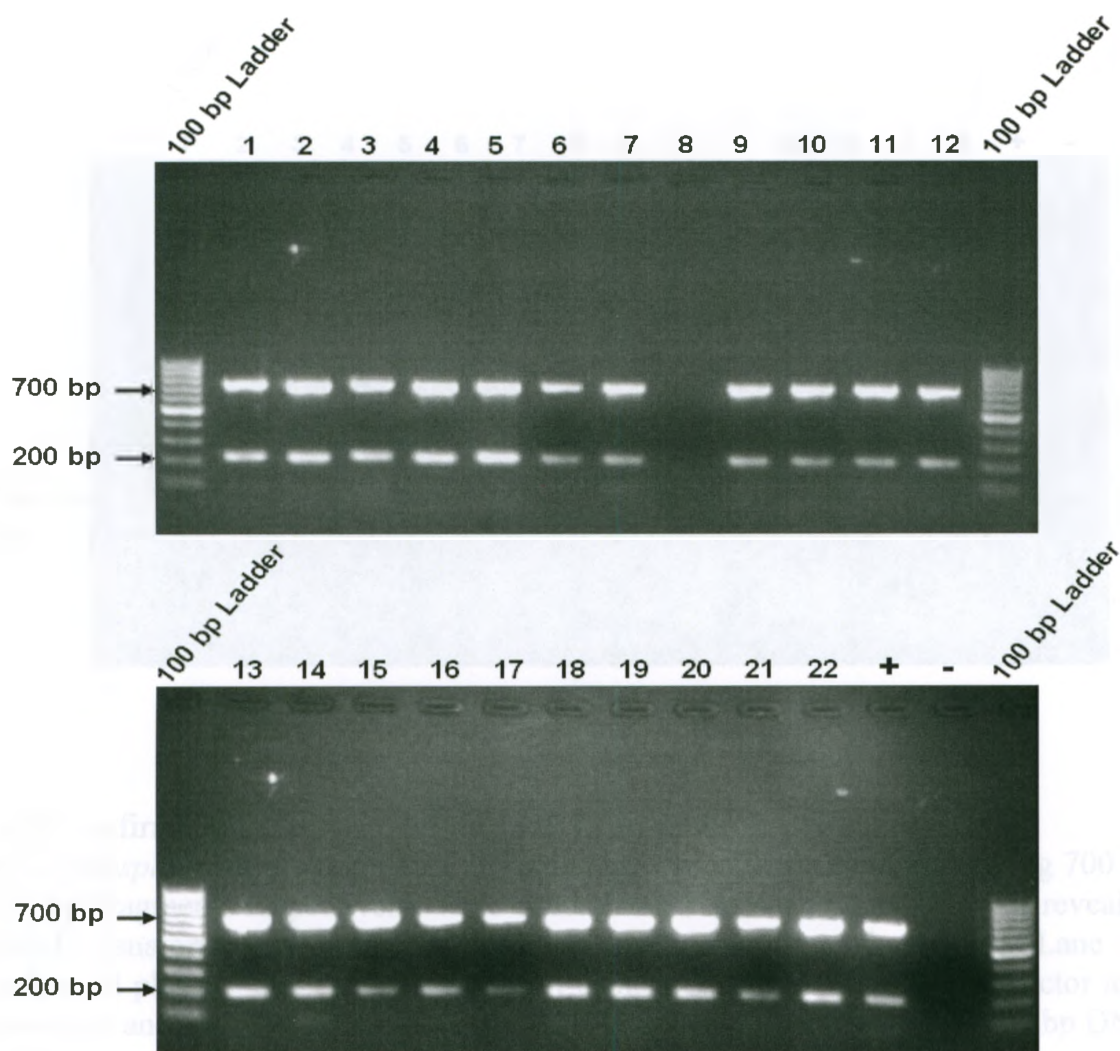


Figure 8. Confirmation of alfalfa carrying *CTB* by multiplex PCR.
Neomycin phosphotransferase II (*NPT II*) and *CTB* specific primers, generating 700 bp and 198 bp fragments, respectively, were used. Lane1 to 7 and lane 9 to 22, PCR products with DNA template from independent transgenic line 1 to 21; Lane 8, untransformed plant; + and -, positive control using pBI-CTB vector as a PCR template and negative control without PCR template, respectively. The 100 bp DNA ladder is indicated.

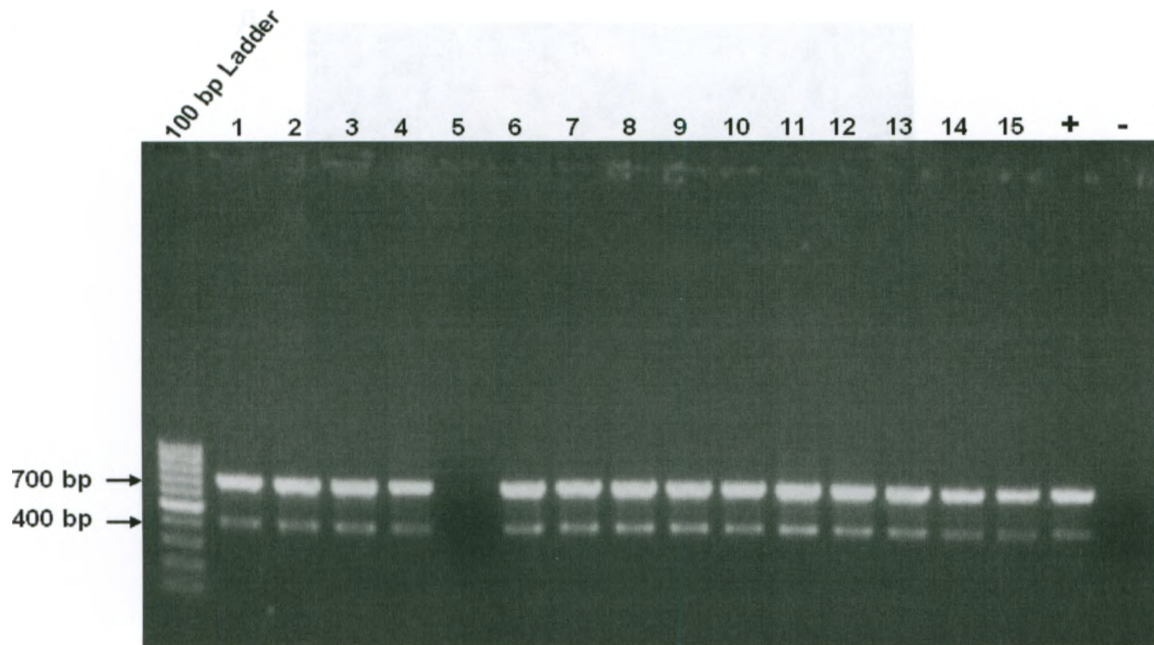


Figure 9. Confirmation of alfalfa carrying *hIL-13* by multiplex PCR.
Neomycin phosphotransferase II (*NPT II*) and *hIL-13* specific primers, generating 700 bp and 415 bp fragments respectively, were used. Lane 1 to 4 and lane 6 to 15, revealed successful transformation from independent transgenic line 1 to 14; Lane 5, untransformed plant; + and -, positive control using pCAMBIA hIL 13-GFP vector as a PCR template and negative control without PCR template, respectively. The 100 bp DNA ladder is indicated.

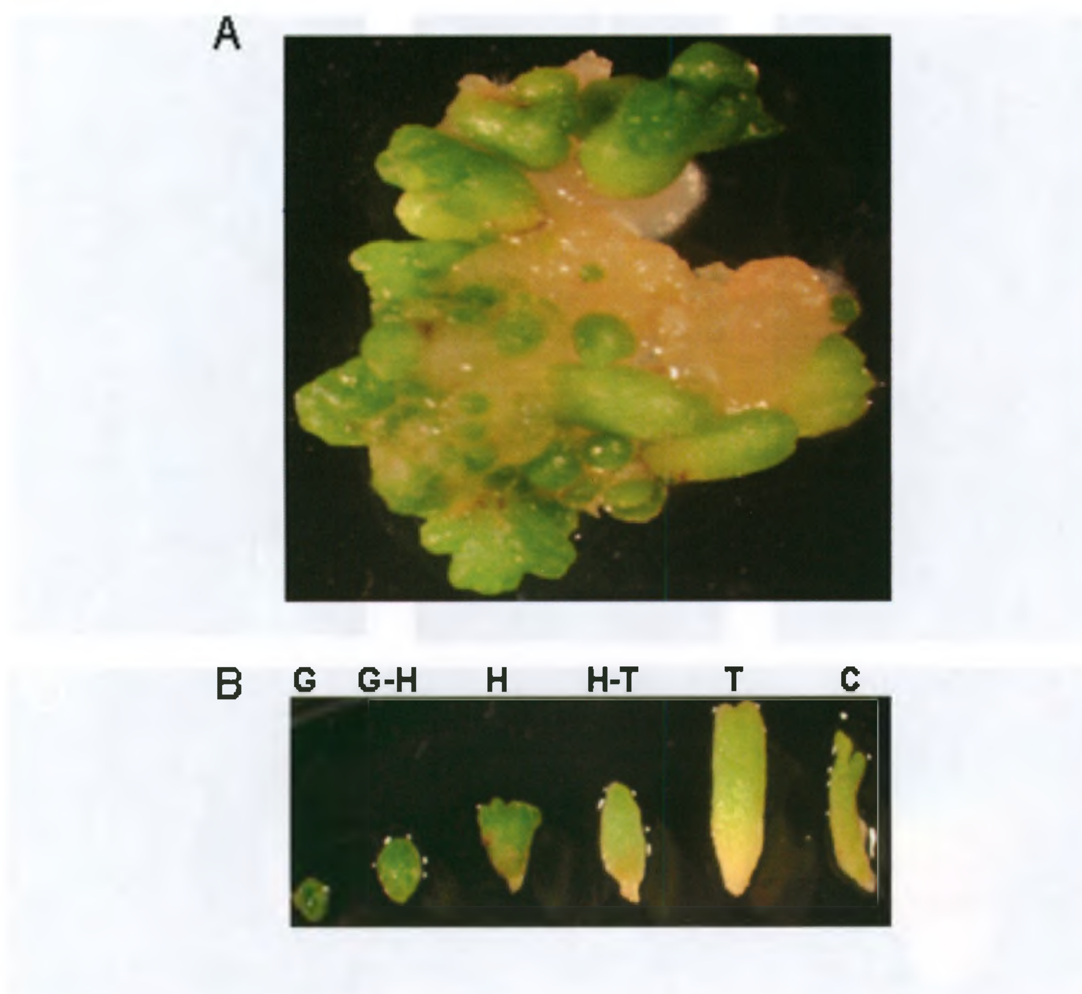


Figure 10. Alfalfa somatic embryo induction in transgenic plants and different developmental stages of somatic embryos.
(A) The green tissues indicate somatic embryos developing from a callus on BOi2Y medium. (B) Different development stages of somatic embryos were shown. G, globular stage; G-H, intermediate growth condition between globular stage and heart stage; H, heart stage; H-T, intermediate growth condition between heart stage and torpedo stage; T, torpedo stage; C, cotyledon stage.

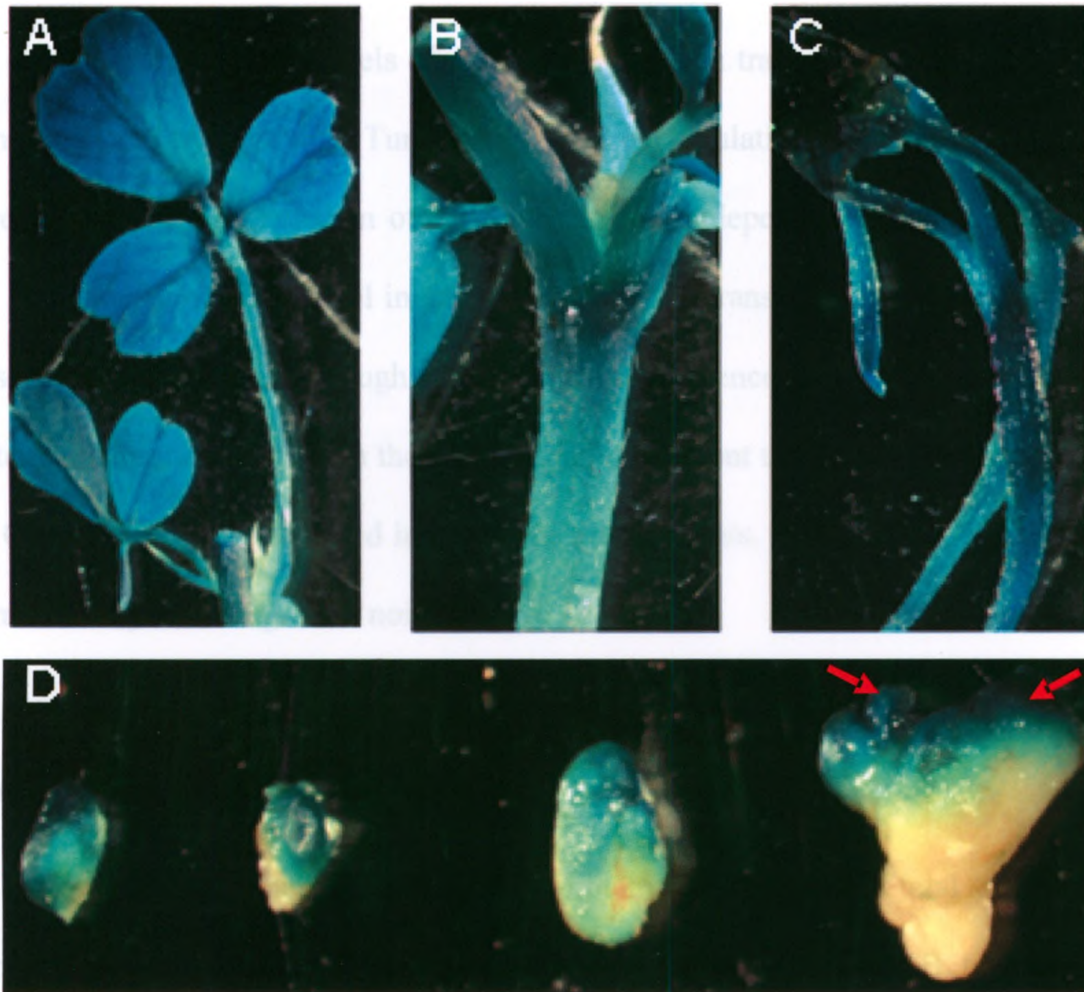


Figure 11. Detection of GUS accumulation in different organs of alfalfa transgenic line 5.

Leaf (A), stem (B), root (C) and somatic embryos (D) all exhibited blue color which indicated GUS protein accumulation in transformed plants. Red arrows showed that blue staining mainly located in cotyledons of transgenic somatic embryos.

and protein accumulation levels of each organ between transgenic lines (Table 3). After being analyzed by following Turkey test, GUS accumulation levels in somatic embryos were significantly higher than other organs in two independent transgenic lines: line 3 and 5. The accumulation level in somatic embryos of transgenic line 12 was higher than those in other organs, although no significant difference was detected. Recombinant protein accumulation levels in the same organ of different transgenic lines were different. No GUS activity was observed in wild type alfalfa plants, the values of wild type alfalfa plants are rely on background noise (Figure 12).

3.4.3 Western blotting for the detection of CTB and hIL-13-GFP fusion protein accumulation

For probing the presence of the CTB protein, thirty five μg of total soluble proteins extracted from different organs of three random independent CTB transgenic lines (Figure 13) and wild type alfalfa plants were resolved by SDS-PAGE and stained by Coomassie blue [Figure 13A-C (a)]. Western blotting analysis detected the existence of the CTB protein [~12 kDa; Figure 13A-C (b)] in all organs of CTB transgenic lines. Similarly, the same amount of total soluble proteins were resolved by SDS-PAGE [Figure 14A-C (a)], and putative non-glycosylated (50 kDa) and glycosylated forms (55 kDa) of hIL-13 [Figure 14A-C (b)] were probed in all organs of three random independent hIL-13 transgenic lines. CTB or hIL-13 proteins cannot be detected in WT lanes. The putative glycosylated form of hIL-13 (based on the higher molecular weight) indicated that biologically active hIL-13 can probably be obtained from transformed alfalfa. The molecular weights of probed proteins were estimated by prestained protein marker on PVDF membrane. The lanes for protein markers were not shown here because

Table 3. Values of two-way ANOVA for analyzing variance among GUS protein accumulation levels in different organs within each alfalfa transgenic line and between transgenic lines expressing GUS.

Expressed Protein	Source of Variation	DF	SS	MS	F	P
GUS	Organs	4	265773036	66443259	61.875	<0.001
	Lines	3	336874560	112291520	104.571	<0.001
	Organs*Lines	12	186298125	15524844	14.457	<0.001

Note: Two-way ANOVA were run using SigmaPlot v11.0 software. DF, degrees of freedom; SS, sum of squares; MS, mean square.

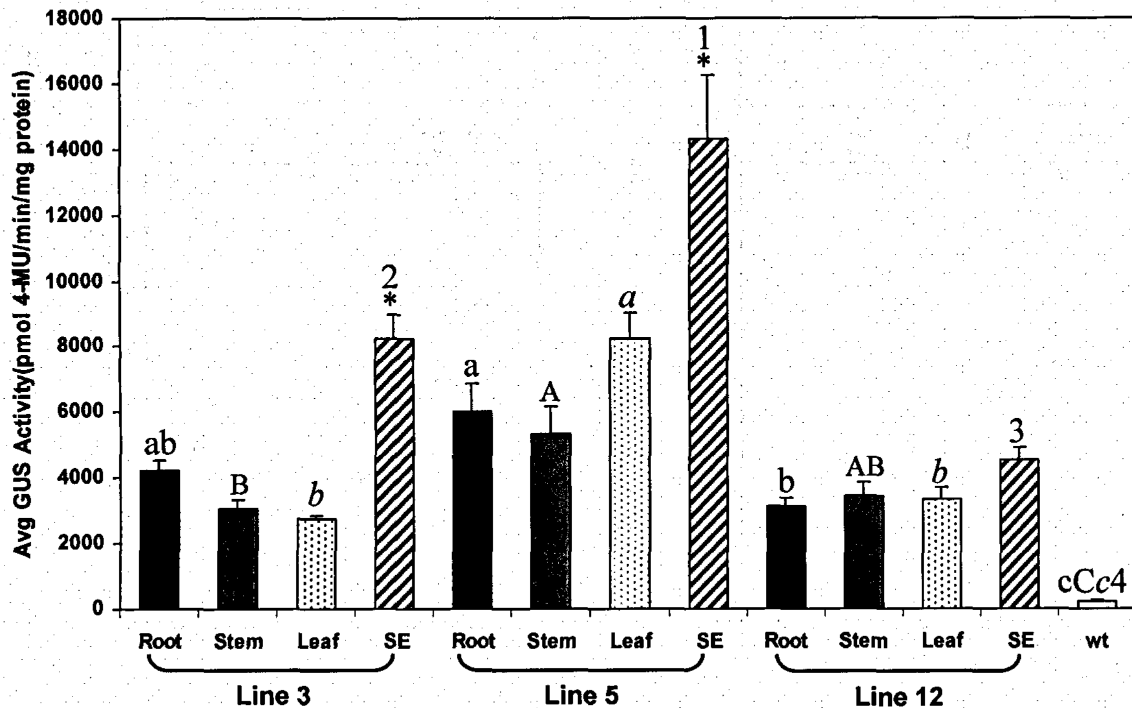


Figure 12. Activity of GUS proteins (pmol 4-MU/min/mg protein) measured by quantitative GUS assay in different organs of three independent alfalfa transgenic lines expressing GUS: line 3, 5, and 12, respectively.

Two hundred μg of total soluble proteins from different organs (root, stem, leaf and somatic embryos) were used to measure the concentration of GUS by quantitative GUS assay. The same amount of total soluble protein from wild type alfalfa was used as a negative control. The assay was performed using three sets of\biological replicate samples and each sample was conducted three times each time. Representative data shown with standard deviation bars was the average of three biologically independent replicates. Statistical analysis was used to determine the significant levels of GUS concentration in somatic embryos versus those in other organs in each transformed line. * denotes GUS concentration values are significantly different in somatic embryos versus those of other organs in the same line. Between lines, different letters or numbers denote significant difference within each organ type-low case represents root, upper case stem, italics leaf, numbers somatic embryos. SE, somatic embryo; wt, wild type alfalfa.

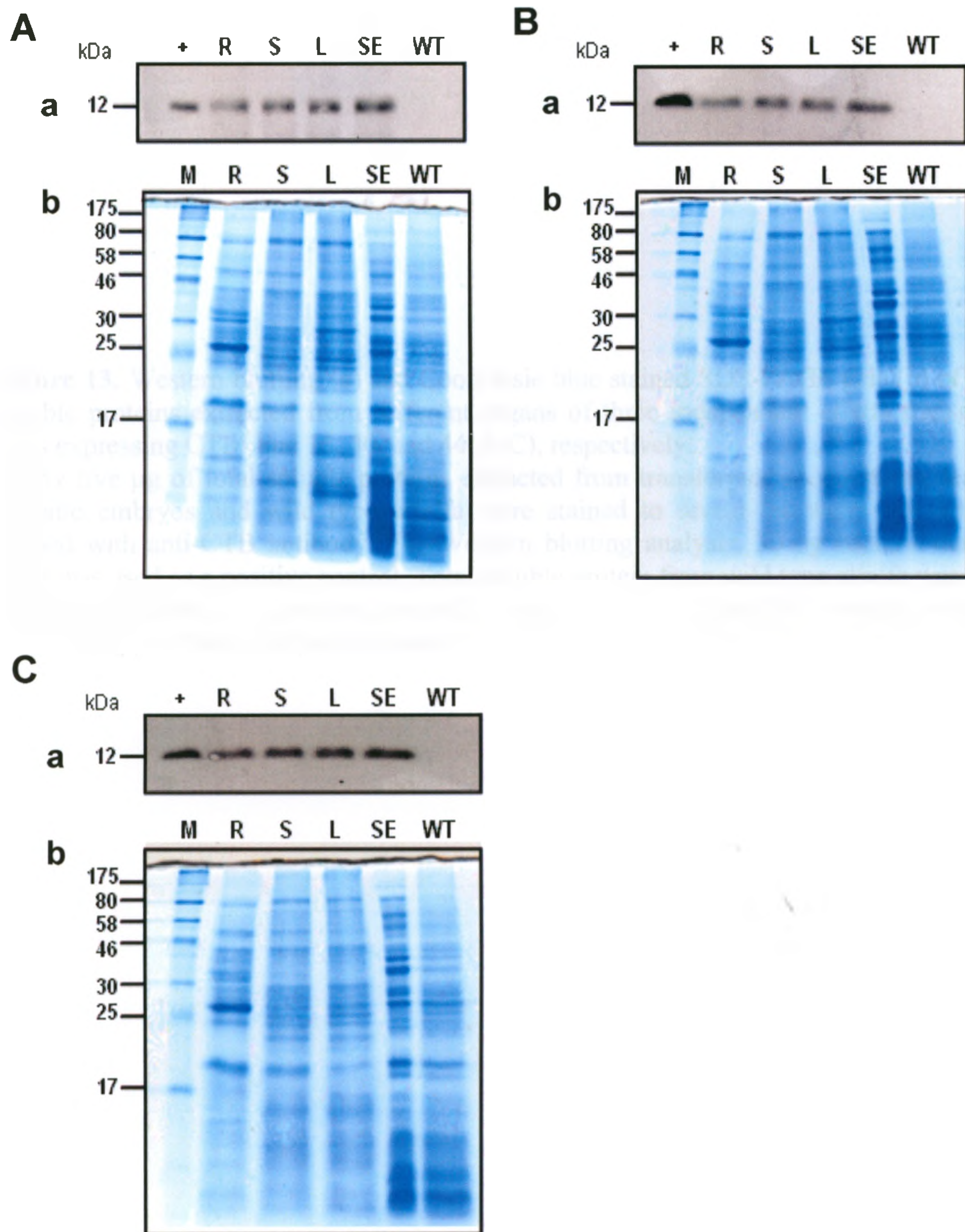
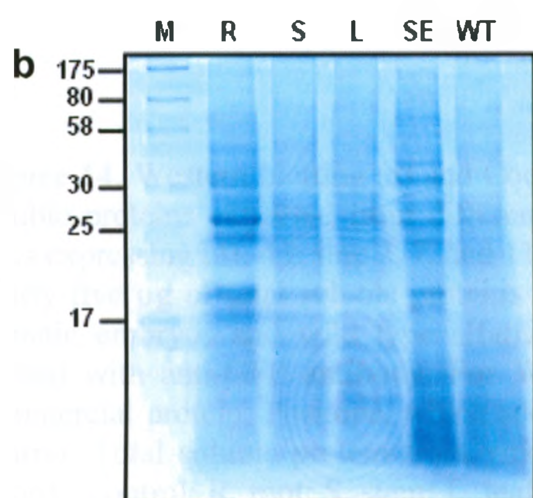
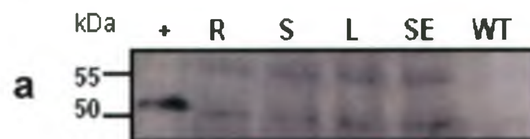

A
B
C
a
b
kDa
+ R S L SE WT
M R S L SE WT
12
175
80
58
46
30
25
17

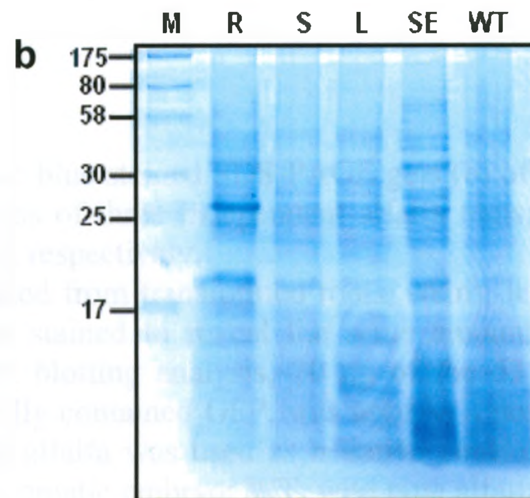
Figure 13. Western blotting (a) and Coomassie blue stained SDS-PAGE gels (b) of total soluble proteins extracted from different organs of three independent alfalfa transgenic lines expressing CTB: line 16, 30, and 44(A-C), respectively.
Thirty five µg of total soluble proteins extracted from transformed roots, stems, leaves, somatic embryos and wild type alfalfa were stained to reveal the same amount and probed with anti-CTB antibody. For Western blotting analysis, 30 ng bacteria-derived CTB was used as a positive control. Total soluble protein from wild type alfalfa was used as negative controls. +, positive control; R, root; S, stem; L, leaf; SE, somatic embryo; WT, wild type alfalfa; M, protein marker.

A



B





C

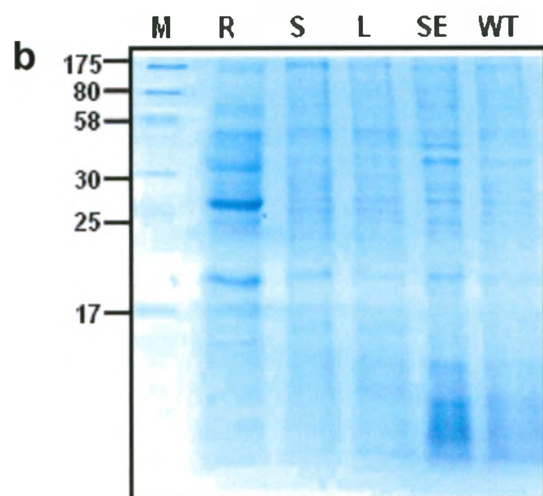


Figure 14. Western blotting (a) and Coomassie blue stained SDS-PAGE gels (b) of total soluble proteins extracted from different organs of three independent alfalfa transgenic lines expressing hIL-13: line 2, 7, and 11(A-C), respectively.
Thirty five μg of total soluble proteins extracted from transformed roots, stems, leaves, somatic embryos and wild type alfalfa were stained to reveal the same amount and probed with anti-GFP antibody. For Western blotting analysis, 20 ng of the 53 kDa commercial protein: Positope, which specifically contained GFP, was used as a positive control. Total soluble protein from wild type alfalfa was used as negative controls. +, positive control; R, root; S, stem; L, leaf; SE, somatic embryo; WT, wild type alfalfa; M, protein marker.

they could not be probed by anti-CTB antibody or anti-GFP antibody.

3.4.4 ELISA for the quantification of CTB and hIL-13-GFP fusion protein

To measure the concentrations of CTB, ELISA was conducted using 100 μg total soluble proteins from different organs of transgenic plants and wild type alfalfa. Readings of series of positive controls, the raw data of which was shown in Supplementary Table 1 in Appendices, were used to plot standard curves. The averages of readings from three technical replicates were calculated and shown in Table 4 to generate standard curves (Figure 15). Readings of samples from three transgenic alfalfa lines expressing CTB were used to determine recombinant protein concentrations based on the equations of standard curves (Supplementary Table 2). Based on three CTB concentrations (three biological replicates) of each samples, the average CTB concentration and standard derivation were calculated and shown in Table 5. Two-way ANOVA, using three CTB concentrations (three biological replicates) of each sample, detected there are significant variances among different organs, different transgenic lines and the interaction between organs and transgenic lines (Table 6). After analyzed by Turkey test, the accumulation levels of heterologous protein CTB in somatic embryos were significantly higher than those in vegetative organs in three transgenic lines: line 16, 30 and 44, and the expression levels in each organ between transgenic lines were significantly different. The average CTB concentration and standard derivation of each sample, and variances among samples were shown in Figure 16. The average CTB accumulation levels in somatic embryos in above three independent transgenic lines reach up to 0.15% of total soluble protein. No CTB protein accumulation was observed in wild type alfalfa plants, the values of wild type alfalfa plants rely on background noise.

Table 4. Average readings for generating standard curves of ELISA to measure CTB concentrations in alfalfa transgenic and wild type plants.

ng/100 μl	CTB biological repeat 1	CTB biological repeat 2	CTB biological repeat 3
0	0.204	0.267	0.193
25	0.507	0.485	0.378
50	0.692	0.617	0.672
75	0.828	0.843	0.792
100	1.075	0.875	0.94
125	1.363	1.213	1.364

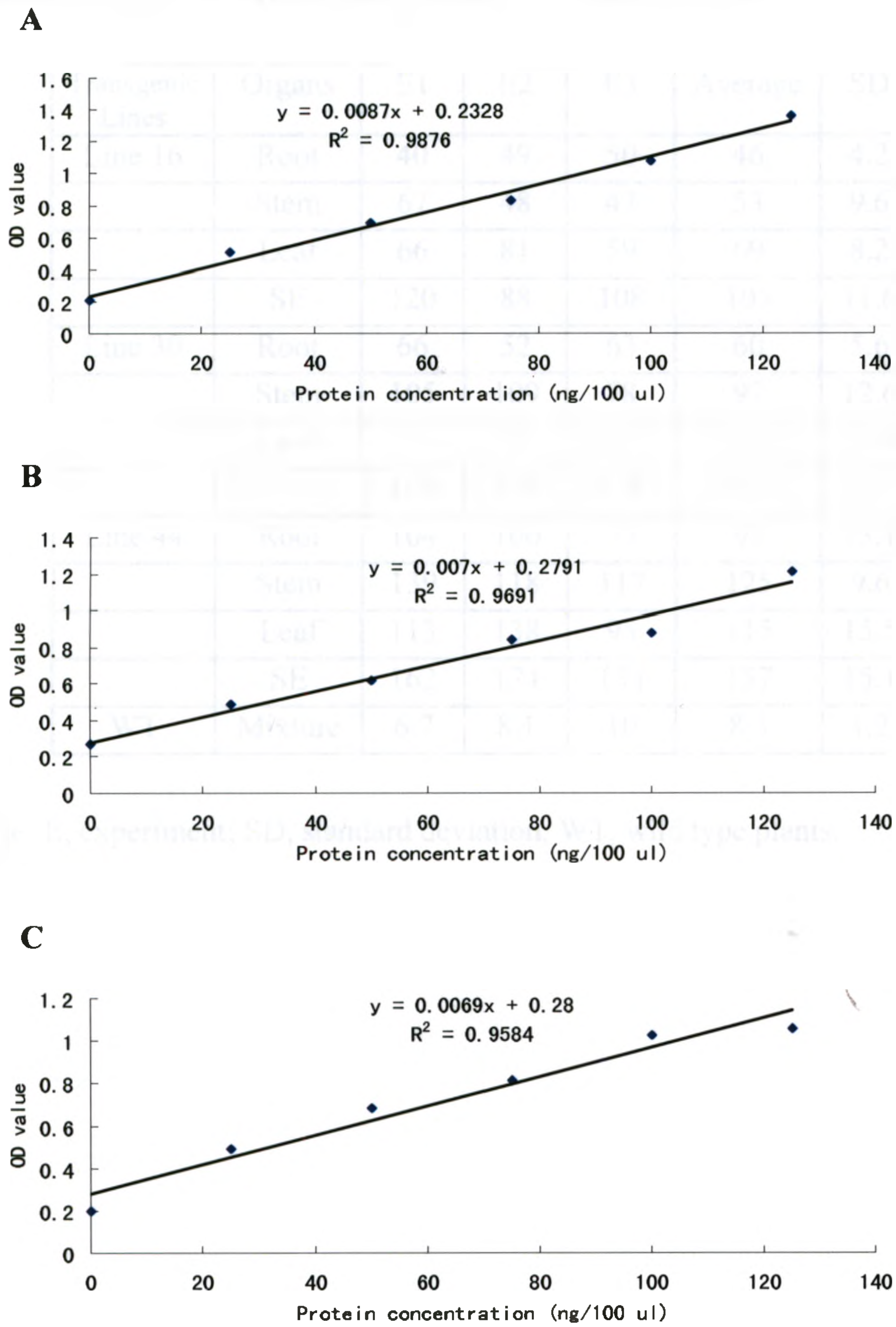


Figure 15. Standard curves of ELISA to measure CTB concentrations in alfalfa transgenic and wild type plants.

A-C, standard curves for three biological replicates.

Table 5. CTB protein concentrations (ng/100 μg) in three biological replicates and their standard deviations in each organ of alfalfa transgenic plants.

Transgenic Lines	Organs	E1	E2	E3	Average	SD
Line 16	Root	40	49	50	46	4.2
	Stem	67	48	43	53	9.6
	Leaf	66	81	59	69	8.2
	SE	120	88	108	105	11.6
Line 30	Root	66	52	63	60	5.6
	Stem	105	109	78	97	12.6
	Leaf	71	75	102	83	12.9
	SE	198	186	158	181	15.1
Line 44	Root	104	100	73	92	13.1
	Stem	139	118	117	125	9.6
	Leaf	113	138	95	115	15.5
	SE	162	174	134	157	15.1
WT	Mixture	6.7	8.1	10	8.3	1.2

Note: E, experiment; SD, standard deviation, WT, wild type plants.

Table 6. Values of two-way ANOVA for analyzing variance among recombinant protein concentrations within each alfalfa transgenic line and between transgenic lines expressing CTB.

Expressed Protein	Source of Variation	DF	SS	MS	F	P
CTB	Organs	4	67666	16916	108.055	<0.001
	Lines	3	76465	25488	162.810	<0.001
	Organs*Lines	12	33569	2797	17.869	<0.001

Note: Two-way ANOVA and Turkey test were run using SigmaPlot v11.0 software. DF, degrees of freedom; SS, sum of squares; MS, mean square.

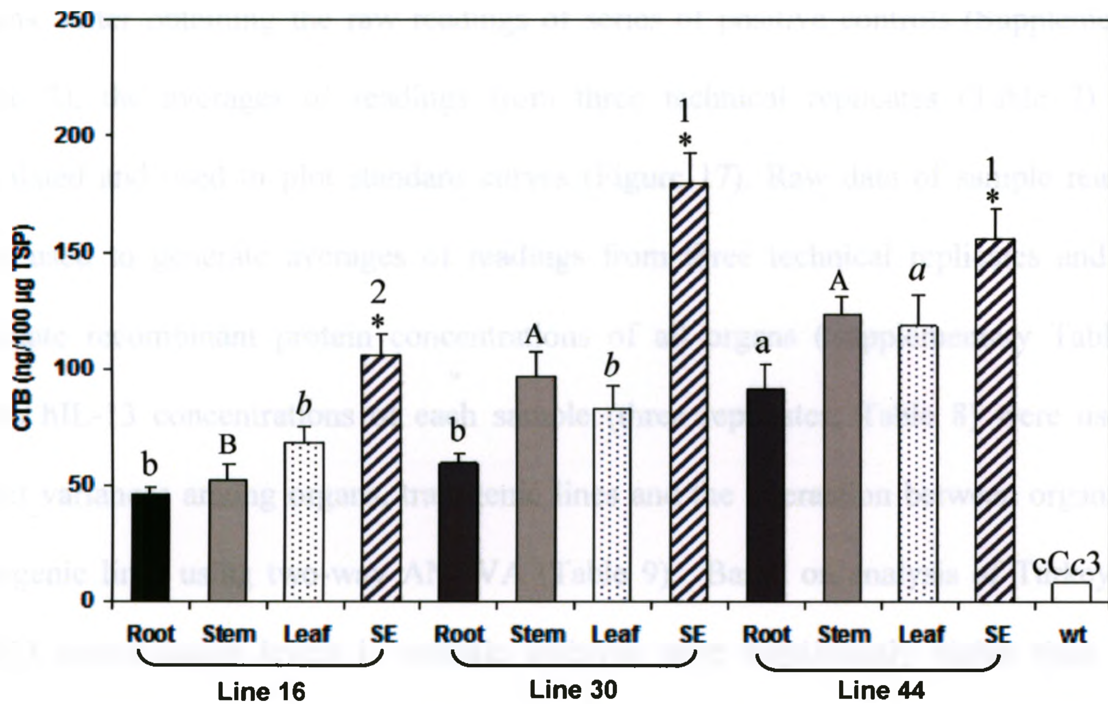


Figure 16. Quantification of CTB concentrations by ELISA in different organs of three independent alfalfa transgenic lines expressing CTB: line 16, 30, and 44, respectively. One hundred µg of total soluble proteins from different organs (root, stem, leaf and somatic embryos) were used to measure the concentration of recombinant CTB by ELISA. The same amount of proteins from wild type alfalfa was used as a negative control. The assay was performed using three sets of biological replicate samples and each sample was conducted repeatedly three times in an assay. Representative data shown with standard deviation bars was the average of three biologically independent replicates. Statistical analysis was used to determine the significant level of CTB concentration in somatic embryos versus those in other organs in each transformed line. * denotes CTB concentration values are significantly different versus those of other organs in the same line. Between lines, different letters or numbers denote significant difference within each organ type-low case represents root, upper case stem, italics leaf, numbers somatic embryos. SE, somatic embryo; wt, wild type alfalfa.

A similar procedure was used to calculate and exhibit hIL-13 concentrations in all organs. After obtaining the raw readings of series of positive controls (Supplementary Table 3), the averages of readings from three technical replicates (Table 7) were calculated and used to plot standard curves (Figure 17). Raw data of sample readings were used to generate averages of readings from three technical replicates and then calculate recombinant protein concentrations of all organs (Supplementary Table 4). Three hIL-13 concentrations of each sample (three replicates; Table 8) were used to detect variances among organs, transgenic lines and the interaction between organs and transgenic lines using two-way ANOVA (Table 9). Based on analysis of Turkey test, hIL-13 accumulation levels in somatic embryos were significantly higher than other organs in two hIL-13 transgenic lines: line 2 and 11. In line 7, the concentration is significantly higher than those in root and stem ($p = 0.001$), and significantly higher than the level in leaf ($p = 0.143$). The average CTB concentration and standard derivation of each sample, and variances among samples were shown in Figure 18. The average hIL-13 accumulation levels in above three independent transgenic somatic embryos reach up to 0.18% of total soluble protein. No hIL-13 protein accumulation was observed in wild type alfalfa plants.

Table 7. Average readings for generating standard curves of ELISA to measure hIL-13 concentrations in alfalfa transgenic and wild type plants.

ng/100 µl	hIL-13 biological repeat 1	hIL-13 biological repeat 2	hIL-13 biological repeat 3
0	0.267	0.146	0.132
25	0.485	0.308	0.325
50	0.617	0.518	0.527
75	0.843	0.827	0.809
100	0.875	0.968	0.975
125	1.213	0.993	1.029

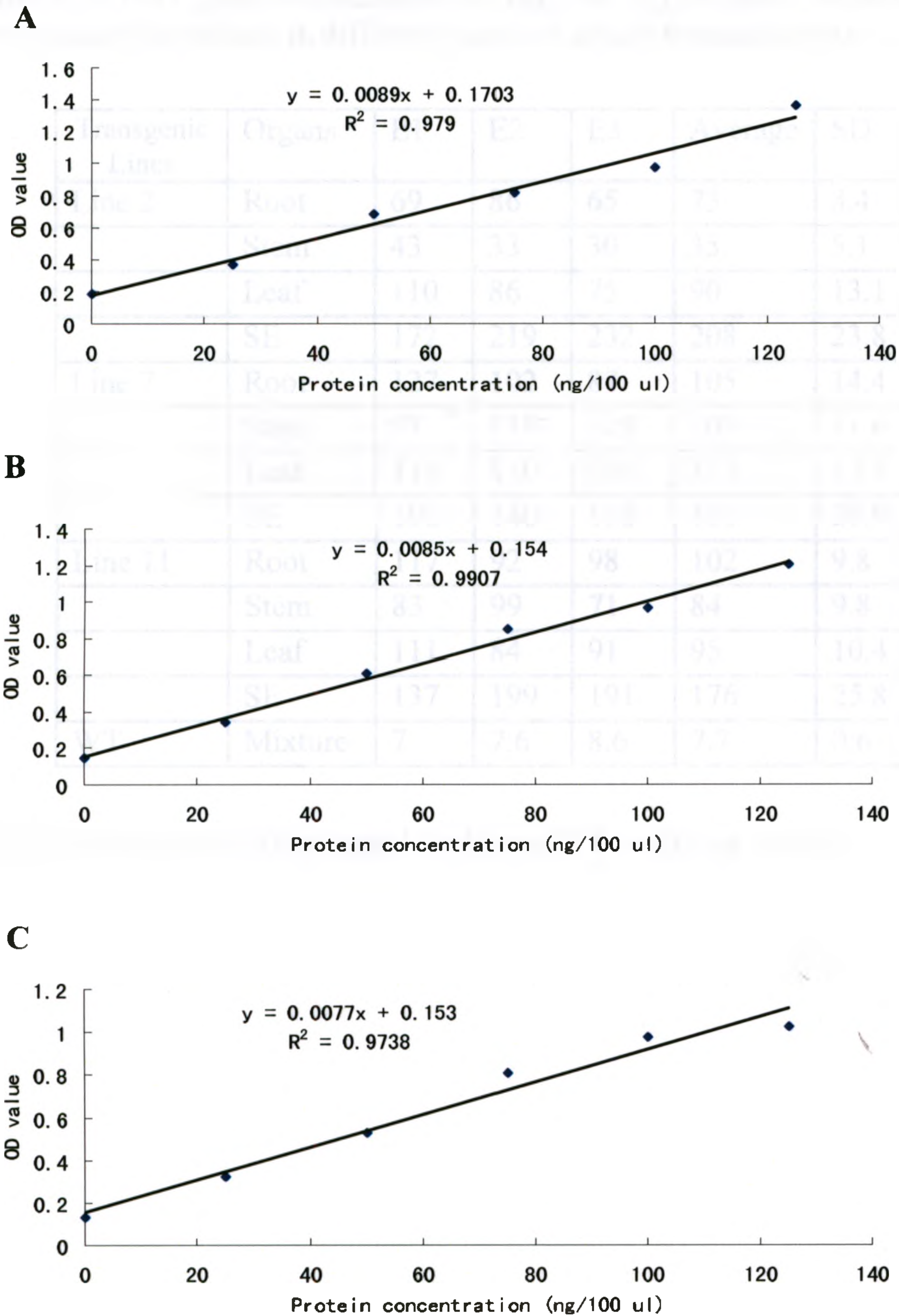


Figure 17. Standard curves of ELISA to measure hIL-13 concentrations in alfalfa transgenic and wild type plants.
A-C, standard curves for three biological replicates.

Table 8. hIL-13 protein concentrations (ng/100 µg) in three biological replicates and their standard deviations in different organs of alfalfa transgenic plants.

Transgenic Lines	Organs	E1	E2	E3	Average	SD
Line 2	Root	69	86	65	73	8.4
	Stem	43	33	30	35	5.1
	Leaf	110	86	75	90	13.1
	SE	172	219	232	208	23.8
Line 7	Root	127	102	87	105	14.4
	Stem	91	119	115	108	11.6
	Leaf	114	130	156	133	15.1
	SE	196	140	158	165	20.9
Line 11	Root	117	92	98	102	9.8
	Stem	83	99	71	84	9.8
	Leaf	111	84	91	95	10.4
	SE	137	199	191	176	25.8
WT	Mixture	7	7.6	8.6	7.7	0.6

Note: E, experiment, SD, standard deviation, WT, wild type plants.

Table 9. Values of two-way ANOVA for analyzing variance among recombinant protein concentrations within each alfalfa transgenic line and between transgenic lines expressing hIL-13.

Expressed Proteins	Source of Variation	DF	SS	MS	F	P
hIL-13	Organs	4	107241	26810	104.987	<0.001
	Lines	3	88080	29360	114.970	<0.001
	Organs*Lines	12	51465	4289	16.794	<0.001

Note: Two-way ANOVA and Turkey test were run using SigmaPlot v11.0 software. DF, degrees of freedom; SS, sum of squares; MS, mean square.

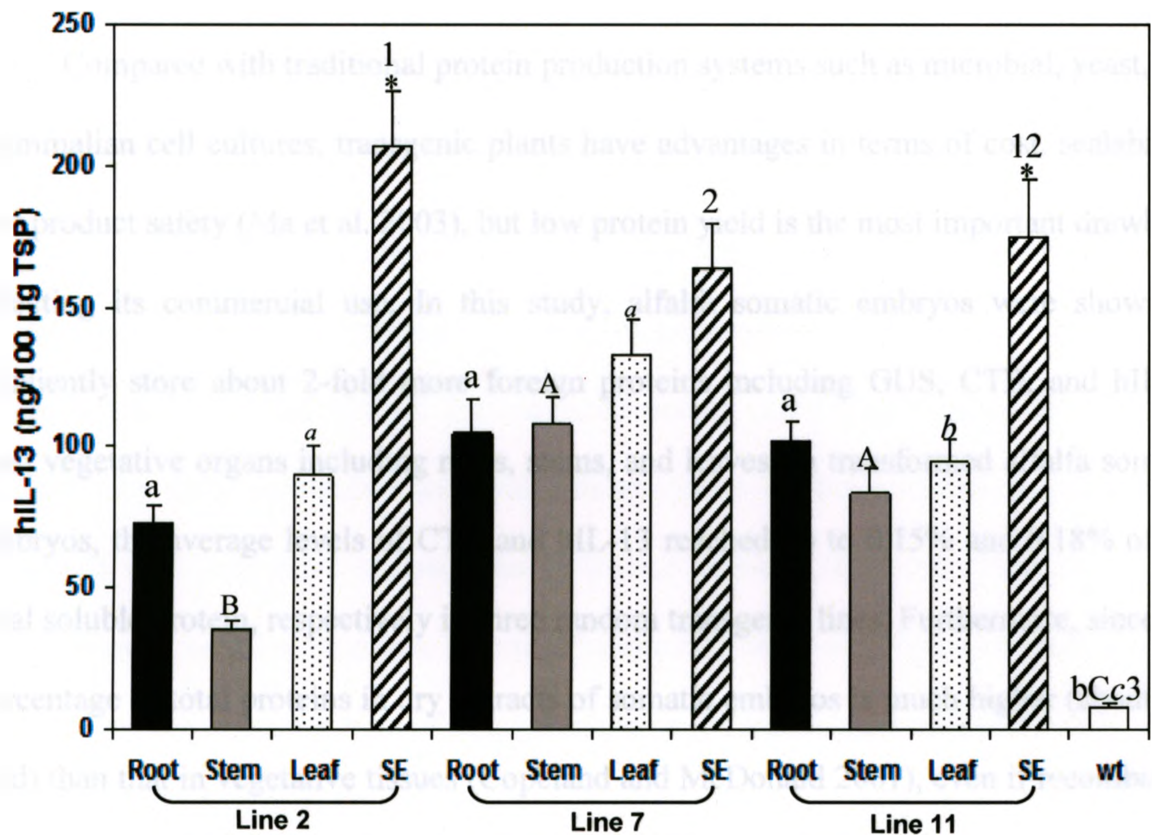


Figure 18. Quantification of hIL-13 concentrations by ELISA in different organs of three independent alfalfa transgenic lines expressing hIL-13: line 2, 7, and 11, respectively. One hundred µg of total soluble proteins from different organs (root, stem, leaf and somatic embryos) were used to measure the concentration of recombinant hIL-13 by ELISA. The same amount of proteins from wild type alfalfa was used as a negative control. The assay was performed using three sets of biological replicate samples and each sample was conducted repeatedly three times in an assay. Representative data shown with standard deviation bars was the average of three biologically independent replicates. Statistical analysis was used to determine the significant level of CTB concentration in somatic embryos versus those in other organs in each transformed line. * denotes hIL-13 concentration values are significantly different versus those of other organs in the same line. Between lines, different letters or numbers denote significant difference within each organ type-low case represents root, upper case stem, italics leaf, numbers somatic embryos. SE, somatic embryo; wt, wild type alfalfa.

CHAPTER 4—DISCUSSION

Compared with traditional protein production systems such as microbial, yeast, and mammalian cell cultures, transgenic plants have advantages in terms of cost, scalability, and product safety (Ma et al. 2003), but low protein yield is the most important drawback affecting its commercial use. In this study, alfalfa somatic embryos were shown to efficiently store about 2-fold more foreign proteins including GUS, CTB, and hIL-13 than vegetative organs including roots, stems, and leaves. In transformed alfalfa somatic embryos, the average levels of CTB and hIL-13 reached up to 0.15% and 0.18% of the total soluble protein, respectively in three random transgenic lines. Furthermore, since the percentage of total proteins in dry extracts of somatic embryos is much higher (about 10-fold) than that in vegetative tissues (Copeland and McDonald 2001), even if recombinant proteins account for the same percentage of total proteins in somatic embryos as in vegetative organs, foreign proteins will be accumulated to about 10-fold higher levels in dry extracts of somatic embryos than in vegetative organs. To my knowledge, this is the first study that shows alfalfa somatic embryo can be used as an efficient expression system to accumulate recombinant proteins. Previous reports have shown that Siberian ginseng somatic embryos can store heterologous proteins (Kang et al. 2006), but no study to date has compared recombinant protein accumulation in somatic embryos versus vegetative organs in plants. The results obtained here are consistent with the hypothesis that alfalfa somatic embryo can efficiently accumulate foreign proteins.

There are significant variance among foreign protein accumulation levels in all four organs within each alfalfa transgenic line, and variance in each organ between transgenic lines, based on statistical analysis. The different abilities of accumulating

recombinant proteins in somatic embryos and vegetative organs could be based on the different roles of plant organs for plant growth. Plant vegetative organs focus on synthesizing and absorbing or transporting all kinds of chemicals for plant growth (Campbell and Reece 2005). It is difficulty for these organs to accumulate a large amount of proteins. In contrast, like zygotic embryos, somatic embryos act as nutrition containers to store large numbers of proteins for embryo germination and early seedling growth (Copeland and McDonald 2001). The enhanced storage capacity of somatic embryos may explain why they accumulate relatively higher yields of heterologous proteins when compared with vegetative organs. The difference between transgenic lines may result from different insertion numbers, insertion location of transgenes in the chromosomes or different growth characteristics of transgenic plants (Filipecki and Malepszy 2006).

In addition to high-level expression of recombinant proteins, there are many other advantages in using somatic embryos. Somatic embryos can be induced for desiccation tolerance by absisic acid (ABA) and form artificial seeds (Senaratna et al. 1990). Under suitable conditions, proteins in artificial seeds, as with normal seeds, remain stable and functional for several years; this is beneficial for storage and transportation (Senaratna 1992). The artificial seed system is a rapid, mass propagation method that maintains genetic fidelity, and allows for direct planting in the greenhouse. This method can propagate thousands of the highest recombinant protein expression lines in a short time, which is beneficial for large scale recombinant protein commercial production (Fujii et al. 1987). Furthermore, leaves, petioles, and stems of alfalfa all can be induced to produce somatic embryos that can increase the yield of somatic embryos of the whole plant (Tian et al. 2002). More importantly, somatic embryos are not used as a food source

and thus minimize regulatory barriers by eliminating the risk of plant-made recombinant proteins entering the food chain.

The accumulation of CTB and hIL-13 in alfalfa somatic embryos was confirmed by Western blotting and ELISA analysis. Interestingly, the hIL-13 was probably glycosylated, a biologically active form, in transformed alfalfa. The anti-GFP antibody detected two bands, 50 kDa and 55 kDa. The 50 kDa band probably represents the unglycosylated form of the hIL-13-GFP fusion protein, while the 55 kDa band may represent the glycosylated form of the hIL-13-GFP fusion protein. Because previous studies have shown that hIL-13 protein contains four potential N-linked glycosylation sites at asparagines 18, 29, 37 and 52 (Minty et al. 1993) and that the molecular weight of glycosylated and unglycosylated hIL-13 differed by 5 kDa (Wang et al. 2008). As a bacteria-derived protein, CTB was probably biologically active in transformed alfalfa like GUS. Almost all reports have shown that plant-synthesized CTB protein retained the ability of specifically binding to GM1-ganglioside, the natural membrane receptor of cholera toxin (Arakawa et al. 1997; Jani et al. 2002; Kim et al. 2006). Future work should include biological activity testing of recombinant proteins hIL-13 and CTB expressed in alfalfa somatic embryos.

CTB and hIL-13 have been expressed in several other plant spices, such as carrot and tobacco. The expression levels of recombinant proteins can differ significantly in different plant species. For example, CTB comprised up to 0.48% of total soluble protein in transgenic carrot (Kim et al. 2009), 0.095% in tobacco (Wang et al. 2001), and 0.15% in alfafa somatic embryo (this study). This indicates that expression levels of CTB in

alfalfa somatic embryos are probably not the highest. Future studies may choose other plants to enhance recombinant protein accumulation in somatic embryos.

It is common to use different aged plant tissues to compare protein accumulation. In this study, two-month-old alfalfa transgenic vegetative tissues and two-week-old somatic embryos were harvested to analyze recombinant protein expression, because different organs have various growth speeds, and plant organs are always collected at their mature stage. Take storage protein accumulation as an example, the accumulation levels of two-week-old alfalfa somatic embryos was used to compare those of mature zygotic embryos, the growth days of which was longer than 14 days (Krochko et al. 1994).

The promoter that is used to control heterologous genes expression in alfalfa somatic embryos needs to be chosen carefully to enhance transcription levels. We used the CaMV 35S promoter to test protein expression pattern as it is one of the most popular promoters used in molecular farming. However, it had been reported that this promoter may not perform as well in alfalfa as it does in other species (Austin and Bingham 1997; Narvaez-Vasquez et al. 1992). Further work choosing other suitable candidate promoters could be performed to enhance recombinant protein production. For example, the light-regulated promoter of the ribulose bisphosphate carboxylase small subunit gene has been shown to have higher driving efficiency in alfalfa than the CaMV 35S promoter (Tabe et al. 1995). The promoter of alfalfa 7S storage protein may also be a good choice because the 7S protein is predominantly expressed in alfalfa somatic embryos (Krochko et al. 1994).

Evaluation of recombinant protein accumulation in alfalfa zygotic embryos was excluded from this study as it takes more than two years for plants to produce them. Most importantly, after cross-pollinating, seeds from transgenic plants would segregate not only the transgenes, but other genes associated with growth and vigour, so that recombinant protein expression levels in zygotic embryos will not be consistent with those in vegetative organs (McKersie et al. 1996). Hence, the protein accumulation levels in zygotic embryos would be highly variable in each transformed line.

Recombinant protein accumulation levels in mixture of alfalfa wild type organs including root, stem, leaf and somatic embryos were used in this study as negative controls, although it is unsuitable for statistical analysis. Theoretically, there is no recombinant protein production in any tissue of wild type alfalfa. But negative controls always were added to make sure accurate experimental handling and to erase background noise using fluorometer. So that, mixture of alfalfa wild type organs rather than all four organs act as negative control in this study.

In summary, recombinant protein production in alfalfa somatic embryos was tested. Three proteins: GUS, CTB, and hIL-13 can significantly accumulate to high levels (around two-fold more recombinant protein accumulation) in alfalfa somatic embryos when compared with vegetative organs. In alfalfa somatic embryos, active GUS protein accumulation was detected by GUS histochemical staining and reconfirmed by quantitative GUS assay. The other foreign proteins CTB and hIL-13 can accumulate up to 0.15% and 0.18% of the total soluble protein, respectively. In addition, the plant-derived hIL-13 protein appeared to be effectively glycosylated to yield biologically active hIL-13.

REFERENCES

Ammirato PV (1983) Embryogenesis: Handbook of Plant Cell Culture. Macmillan Publishing Co, New York, USA, pp 82-123.

Arakawa T, Chong DK, Merritt JL, Langridge WH (1997) Expression of cholera toxin B subunit oligomers in transgenic potato plants. Transgenic Res 6:403-413.

Austin S, Bingham ET (1997) The potential use of transgenic alfalfa as a bioreacotor for the production of industrial enzymes: Biotechnology and the Improvement of Forage Legumes, CAB International, Wallingford, UK, pp 409-427.

Bancroft AJ, McKenzie AN, Grencis RK (1998) A critical role for IL-13 in resistance to intestinal nematode infection. J Immunol 160:3453-3461.

Bardor M, Loutelier-Bourhis C , Paccalet T, Cosette P, Fitchette AC, Vézina LP, Trépanier S, Dargis M, Lemieux R, Lange C, Faye L, Lerouge P (2003) Monoclonal C5-1 antibody produced in transgenic alfalfa plants exhibits a N-glycosylation that is homogenous and suitable for glyco-engineering into human-compatible structures. Plant Biotechnol J 1:451-462.

Barta A, Sommergruber K, Thompson D, Hartmuth K, Matzke MA, Matzke AJM (1986) The expression of a nopaline synthase-human growth hormone chimaeric gene in transformed tobacco and sunflower callus tissue. Plant Mol Biol 6:347-357.

Bevan M (1984) Binary *Agrobacterium* vectors for plant transformation. Nucleic Acids Res 12:8711-8721.

Blaydes DF (1966) Interaction of kinetin and various inhibitors in the growth of soybean tissues. Physiol Plant 19:748-753.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analyt Biochem 72:248-254.

Busse U, Levée V, Trépanier S, Vézina L (2001) Production of antibodies in alfalfa (*Medicago sativa*): Molecular Farming of Plants and Animal for Human and Veterinary Medicine. J Wiley & Sons, New York, USA, pp 199-219.

Campbell NA, Reece JB (2005) Plant structure, growth, and development: Biology. Pearson Education Inc., San Francisco, USA, pp 712-717.

Cannon-Carlson S, Varnerin J, Tsarbopoulos A, Jenh CH, Cox MA, Chou CC, Connelly N, Zavodny P, Tang JCT (1998) Expression, purification, and characterization of recombinant human interleukin-13 from NS-0 cells. Protein Expr Purif 12:239-248.

Chamberlain JS, Gibbs RA, Ranier JE, Nguyen PN, Caskey CT (1988) Deletion screening of the Duchenne muscular dystrophy locus via multiplex DNA amplification. Nucleic Acids Res 16:11141-11156.

Conley AJ, Mohib K, Jevnikar AM, Brandle JE (2009) Plant recombinant erythropoietin attenuates inflammatory kidney cell injury. Plant Biotechnol J 7:183-199.

Copeland LO, McDonald MB (2001) Seed science and technology. Kluwer, Norwell, USA, pp 32-56.

Daniell H, Streatfield SJ, Wycoff K (2001) Medical molecular farming: production of antibodies, biopharmaceuticals and edible vaccines in plants. Trends Plant Sci 6: 219-226.

Deak M, Kiss GB, Koncz C, Dudits D (1986) Transformation of *Medicago* by *Agrobacterium* mediated gene transfer. Plant Cell Rep 5:97-100.

De Zoeten GA, Penswick JR, Horisberger MA, Ahl P, Schultze M, Hohn T (1989) The expression, localization, and effect of a human interferon in plants. Virology 172:213-222.

Dodeman VL, Ducreux G, Kreis M (1997) Zygotic embryogenesis versus somatic embryogenesis. J Exp Bot 48:1493-1509.

Du S, Erickson L and Bowley S (1994) Effect of plant genotype on the transformation of cultivated alfalfa (*Medicago sativa*) by *Agrobacterium tumefaciens*. Plant Cell Rep 13:330-334.

Düring K, Hippe S, Kreuzaler F, Schell J (1990) Synthesis and self-assembly of a functional monoclonal antibody in transgenic Nicotiana tabacum. Plant Mol Biol 15:281-293.

Filipecki M, Malepszy S (2006) Unintended consequences of plant transformation: a molecular insight. J Appl Genet 47:277-286.

Fraley RT, Rogers SG, Horsch RB, Sanders PR, Flick JS, Adams SP, Bittner ML, Brand LA, Fink CL, Fry JS, Galluppi GR, Goldberg SB, Hoffmann NL, Woo SC (1983) Expression of bacterial genes in plant cells. Proc Natl Acad Sci USA 80:4803-4807.

Fujii JA, Slade DT, Redenbaugh K, Walker KA (1987) Artificial seeds for plant propagation. Trends Biotechnol 5:335-339.

Harwood JL (1997) Plant lipid metabolism: Plant Biochemistry. Academic Press, San Diego, USA, pp 237-272.

Herman EM, Larkins BA (1999) Protein storage bodies and vacuoles. Plant Cell 11:601-613.

Hiatt A, Cafferkey R, Bowdish K (1989) Production of antibodies in transgenic plants. Nature 342:76-78.

Holmgren J, Lycke N, Czerkinsky C (1993) Cholera toxin and cholera B subunit as oral-mucosal adjuvant and antigen vector systems. Vaccine 11:1179-1184.

Hooykaas PJJ, Schilperoort RA (1992) *Agrobacterium* and plant genetic engineering. Plant Mol Biol 19:15-38.

Jaiwal PK, Sautter C, Potrykus I (1998) *Agrobacterium rhizogenes* mediated gene transfer in mung bean (*Vigna radiata* (L.) Wilczek). Current Sci 75:41-45.

Jani D, Meena LS, Rizwan-ul-Haq QM, Singh Y, Sharma AK, Tyagi AK (2002) Expression of cholera toxin B subunit in transgenic tomato plants. Transgenic Res 11:447-454.

Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS-fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J 6:3901-3907.

Kang TJ, Lee WS, Choi EG, Kim JW, Kim BG, Yang MS (2006) Mass production of somatic embryos expressing *Escherichia coli* heat-labile enterotoxin B subunit in Siberian ginseng. Biotechnol J 121:124-133.

Kay R, Chan A, Daly M, McPherson J (1987) Duplication of CaMV 35S promoter sequences creates a strong enhancer for plant genes. Science 236:1299-1302.

Khan MS, Maliga P (1999) Fluorescent antibiotic resistance marker for tracking plastid transformation in higher plants. Nature Biotechnol 17:910-915.

Khoudi H, Laberge S, Ferullo JM, Bazin R, Darveau A, Castonguay Y, Allard G, Lemieux R, Vézina LP (1999) Production of a diagnostic monoclonal antibody in perennial alfalfa plants. Biotechnol Bioeng 64:135-143.

Kim YS, Kim BG, Kim TG, Kang TJ, Yang MS (2006) Expression of a cholera toxin B subunit in transgenic lettuce (*Lactuca sativa* L.) using *Agrobacterium*-mediated transformed system. Plant Cell Tissue Organ Cult 87:203-210.

Kim YS, Kim MY, Kim TG, Yang MS (2009) Expression and assembly of cholera toxin B subunit (CTB) in transgenic carrot (*Daucus carota* L.). Mol Biotechnol 41:8-14.

Krochko JE, Bewley JD (1988) Use of electrophoretic techniques in determining the composition of seed storage proteins in alfalfa. Electrophoresis 9:751-763.

Krochko JE, Bantroch DJ, Greenwood JS, Bewley JD (1994) Seed storage proteins in developing somatic embryos of alfalfa: defects in accumulation compared to zygotic embryos. J Exp Bot 45:699-708.

Lau OS, Sun SSM (2009) Plant seeds as bioreactors for recombinant protein production. Biotechnol Adv 27: 1015-1022.

Li D, O'Leary J, Huang Y, Huner NP, Jevnikar AM, Ma S (2006) Expression of cholera toxin B subunit and the B chain of human insulin as a fusion protein in transgenic tobacco plants. Plant Cell Rep 25:417-424.

Ma JK, Drake PM, Christou P (2003) The production of recombinant pharmaceutical proteins in plants. Nat Rev Genet 4:794-805.

Maheswaran G, Williams EG (1984) Direct somatic embryoid formation on immature embryos of *Ttifolium repens, T. pratense* and *Medicago sativa* and rapid clonal propagation of T. *repens.* Ann Bot 54:201-211.

McCormick SJ, Tjian R (2010) A new focus on plant sciences. Science 330:1021.

McGhee JR, Mestecky J, Dertzbaugh MT, Eldridge JH, Hirasawa M, Kiyono H (1992) The mucosal immune system: From fundamental concepts to vaccine development. Vaccine 10: 75-88.

McKersie BD, Bowley SR, Harjanto E, Leprince O (1996) Water-deficit tolerance and field performance of transgenic alfalfa overexpressing superoxide dismutase. Plant Physiol 111:1177-1181.

McKersie BD, Murnagham KS, Jones KS, Bowley SR (2000) Iron-superoxide dismutase expression in transgenic alfalfa increases winter survival without a detectable increase in photosynthetic oxidative stress tolerance. Plant Physiol 122:1427-1437.

Meinke DW, Chen J, Beachy RN (1981) Expression of storage-protein genes during soybean seed development. Planta 153:130-139.

Minty A, Chalon P, Derocq JM, Dumont X, Guillemot JC, Kaghad M, Labit C, Leplatois P, Liauzun P, Miloux B (1993) lnterleukin-13 is a new human lymphokine regulating inflammatory and immune responses. Nature 362:248-250.

Montaner LJ, Doyle AG, Collin M, Herbein G, Illei P, James W, Minty A, Caput D, Ferrara P, Gordon S (1993) Interleukin 13 inhibits human immunodeficiency virus type 1 production in primary blood-derived human macrophages in vitro. J Exp Med 178:743-747.

Munro S, Pelham HR (1987) A C-terminal signal prevents secretion of luminal ER proteins. Cell 48:899-907.

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473-497.

Namasivayam P (2007) Acquisition of embryogenic competence during somatic embryogenesis. Plant Cell Tissue Organ Cult 90:1-8.

Narvaez-Vasquez J, Orozco-Cardenas ML, Ryan CA (1992) Differential expression of a chimeric CaMV-tomato proteinase Inhibitor I gene in leaves of transformed nightshade, tobacco and alfalfa plants. Plant Mol Biol 20:1149-1157.

Odell JT, Nagy F, Chua NH (1985) Identification of DNA sequences required for activity of the cauliflower mosaic virus 35S promoter. Nature 313:810-812.

Petruccelli S, Otegui MS, Lareu F, Tran Dinh O, Fitchette AC, Circosta A, Rumbo M, Bardor M, Carcamo R, Gomord V, Beachy RN (2006) A KDEL-tagged monoclonal antibody is efficiently retained in the endoplasmic reticulum in leaves, but is both partially secreted and sorted to protein storage vacuoles in seeds. Plant Biotechnol J 4:511-527.

Schenk RU, Hildebrandt AC (1972) Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. Can J Bot 50:199-204.

Schillberg S, Fischer R, Emans N (2003) Molecular farming of recombinant antibodies in plants. Cell Mol Life Sci 60:433-445.

Senaratna T, McKersie BD, Bowley SR (1990) Artificial seeds of alfalfa (*Medicago sativa* L.) induction of desiccation tolerance in somatic embryos. *In Vitro* Cell Dev Biol 26:85-90.

Senaratna T (1992) Artificial seeds. Biotech Adv 20:379-392.

Shahin EA, Spielmann A, Suhkapinda K, Simpson RB, Yasher M (1986) Transformation of cultivated alfalfa using disarmed *Agrobacterium tumefaciens*. Crop Sci 26:1235-1239.

Shewry PR, Napier JA, Tatham AS (1995) Seed storage protein: structures and biosynthesis. Plant Cell 7:945-956.

Shimamura T, Husain SR, Puri RK (2006) The IL-4 and IL-13 pseudomonas exotoxins: new hope for brain tumor therapy. Neurosurg Focus 20:E11.

Shimomura O, Johnson FH, Saiga Y (1962) Extraction, purification and properties of aequorin, a bioluminescent protein from the luminous hydromedusan, aequorea. J Cell Comp Physiol 59:223-239.

Sijmons PC, Dekker BMM, Schrammeijer B, Verwoerd TC, van den Elzen PJM van den, Hoekema A (1990) Production of correctly processed human serum albumin in transgenic plants. Nat Biotechnol 8:217-221.

Steward FC, Mapes MO, Mears K (1958) Growth and organized development of cultured cells. II. organization in cultures from freely suspended cells. Amer J Bot 45:705-708.

Svab Z, Hajdukiewicz P, Maliga P (1990) Stable transformation of plastids in higher plants. Proc Natl Acad Sci USA, 87:8526-8530.

Swartz JR (2001) Advances in *Escherichia coli* production of therapeutic proteins. Curr Opin Biotechnol 12:195-201.

Tabe LM, Wardley-Richardson T, Ceriotti A, Aryan A, McNabb W, Moore A, Higgins TJ (1995) A biotechnological approach to improving the nutritive value of alfalfa. J Anim Sci 73:2752-2759.

Taiz L, Zeiger E (1998) Plant Physiology. Sinauer Associates Inc., Sunderland, USA, pp 155-456.

Tesfaye M, Temple SJ, Allan DL, Vance CP, Samac DA (2001) Overexpression of malate dehydrogenase in transgenic alfalfa enhances organic acid synthesis and confers tolerance to aluminum. Plant Physiol 127:1836-1844.

Tesfaye M, Denton MD, Samac DA, Vance CP (2005) Transgenic alfalfa secretes a fungal endochitinase protein to the rhizosphere. Plant Soil 269:233-243.

Thompson JP, Debinski W (1999) Mutants of interleukin 13 with altered reactivity toward interleukin 13 receptors. J Biol Chem 274:29944-29950.

Tian L, Wang H, Wu K, Latoszek-Green M, Hu M, Miki B, Brown D (2002) Efficient recovery of transgenic plants through organogenesis and embryogenesis using a cryptic promoter to drive marker gene expression. Plant Cell Rep 20:1181-1187.

Tregoning JS, Nixon P, Hiroshi K, Svab Z, Clare S, Bowe F, Fairweather N, Ytterberg J, van Wijk KJ, Dougan G, Maliga P (2003) Expression of tetanus toxin Fragment C in tobacco chloroplasts. Nucleic Acids Res 31:1174-1179.

Twyman RM, Stöger E, Schillberg S, Christou P, Fischer R (2003) Molecular farming in plants: host systems and expression technology. Trends Biotechnol 21:570-578.

Twyman RM, Schillberg S, Fischer R (2005) Transgenic plants in the biopharmaceutical market. Expert Opin Emerg Drugs 10:185-218.

Wang DJ, Brandsma M, Yin Z, Wang A, Jevnikar AM, Ma S (2008) A novel platform for biologically active recombinant human interleukin-13 production. Plant Biotechnol J 6:504-515.

Wang XG, Zhang GH, Liu CX, Zhang YH, Xiao CZ, Fang RX (2001) Purified cholera toxin B subunit from transgenic tobacco plants possesses authentic antigenicity. Biotechnol Bioeng 72:490-494.

Wilde HD, Nelson WS, Booij H, Vries SC, Thomas TL (1988) Gene-expression programs in embryogenic and non-embryogenic carrot cultures. Planta 176:205-211.

Winkelmann T, Heintz D, Van Dorsselaer A, Serek M, Braun HP (2006) Proteomic analyses of somatic and zygotic embryos of *Cyclamen persicum* Mill. reveal new insights into seed and germination physiology. Planta 224:508-519.

Ziegelhoffer T, Will J, Austin-Phillips S (1999) Expression of bacterial cellulase genes in transgenic alfalfa (*Medicago sativa* L.), potato (*Solanum tuberosum* L.) and tobacco (*Nicotiana tabacum* L.). Mol Breed 5:309-318.

APPENDICES

Supplementary Table 1. Raw data of reading for standard curves of ELISA to measure CTB concentrations in alfalfa transgenic and wild type plants.

Positive control ng/100 ul	Experiment 1			Average
0	0.202	0.209	0.202	0.204
20	0.515	0.501	0.504	0.507
50	0.69	0.697	0.688	0.692
75	0.834	0.818	0.833	0.828
100	1.075	1.079	1.071	1.075
125	1.354	1.364	1.372	1.363
	Experiment 2			Average
0	0.264	0.269	0.267	0.267
20	0.479	0.491	0.484	0.485
50	0.602	0.615	0.634	0.617
75	0.821	0.839	0.868	0.843
100	0.927	0.864	0.835	0.875
125	1.107	1.221	1.312	1.213
	Experiment 3			Average
0	0	0.201	0.175	0.193
20	0.481	0.49	0.362	0.378
50	0.662	0.697	0.685	0.672
75	0.816	0.807	0.837	0.792
100	1.004	1.037	0.945	0.94
125	1.036	1.07	1.322	1.364

Supplementary Table 2. Raw data of sample readings and protein concentration calculation of ELISA to measure CTB concentrations in alfalfa transgenic and wild type plants.

Root Line 16	R1	R2	R3	Average	Protein C. (ng/ 100 μg)
E1	0.579	0.568	0.585	0.577	40
E2	0.629	0.625	0.634	0.629	50
E3	0.61	0.614	0.625	0.616	49
Stem Line 16					
E1	0.814	0.817	0.819	0.817	67
E2	0.606	0.622	0.617	0.615	48
E3	0.563	0.589	0.571	0.574	43
Leaf Line 16					
E1	0.823	0.816	0.808	0.816	66
E2	0.851	0.841	0.845	0.846	81
E3	0.696	0.685	0.699	0.693	59
SE Line 16					
E1	1.282	1.279	1.271	1.277	120
E2	0.903	0.894	0.887	0.895	88
E3	1.015	1.037	1.034	1.028	108
Root Line 30					
E1	0.816	0.802	0.804	0.807	66
E2	0.647	0.632	0.649	0.643	52
E3	0.715	0.706	0.721	0.714	63
Stem Line 30					
E1	1.149	1.144	1.145	1.146	105
E2	1.051	1.036	1.038	1.042	109
E3	0.825	0.804	0.827	0.819	78
Leaf Line 30					
E1	0.85	0.847	0.855	0.851	71
E2	0.801	0.802	0.808	0.804	75
E3	0.997	1.003	0.983	0.994	102
SE Line 30					
E1	1.971	1.937	1.958	1.955	198
E2	1.585	1.567	1.592	1.581	186
E3	1.376	1.372	1.367	1.372	158

Root Line 44	R1	R2	R3	Average	Protein C. (ng/ 100 μg)
E1	1.137	1.152	1.124	1.138	104
E2	0.979	0.992	0.965	0.979	100
E3	0.789	0.792	0.772	0.784	73
Stem Line 44					
E1	1.431	1.44	1.456	1.442	139
E2	1.004	1.129	1.183	1.105	118
E3	1.095	1.081	1.078	1.085	117
Leaf Line 44					
E1	1.211	1.225	1.213	1.216	113
E2	1.246	1.259	1.231	1.245	138
E3	0.927	0.935	0.953	0.938	95
SE Line 44					
E1	1.631	1.649	1.647	1.642	162
E2	1.643	1.35	1.998	1.497	174
E3	1.195	1.206	1.212	1.204	134
WT					
E1	0.292	0.295	0.285	0.291	6.7
E2	0.323	0.339	0.345	0.336	8.1
E3	0.342	0.348	0.356	0.349	10

Note: E, experiment; R, repeat; Protein C., protein concentration; SE, somatic embryo; WT, wild type alfalfa.

Supplementary Table 3. Raw data of readings for standard curves of ELISA to measure hIL-13 concentrations in alfalfa transgenic and wild type plants.

Positive control ng/100 µl	Experiment 1			Average
0	0.175	0.193	0.267	0.267
20	0.362	0.378	0.484	0.485
50	0.685	0.672	0.634	0.617
75	0.837	0.792	0.868	0.843
100	0.945	0.94	0.835	0.875
125	1.322	1.364	1.312	1.213
	Experiment 2			Average
0	0.152	0.134	0.121	0.146
20	0.341	0.349	0.352	0.308
50	0.621	0.562	0.542	0.518
75	0.869	0.857	0.785	0.827
100	0.973	0.985	0.962	0.968
125	1.224	1.175	1.055	0.993
	Experiment 3			Average
0	0.121	0.146	0.128	0.132
20	0.352	0.308	0.314	0.325
50	0.542	0.518	0.52	0.527
75	0.785	0.827	0.814	0.809
100	0.962	0.968	0.996	0.975
125	1.055	0.993	1.039	1.029

Supplementary Table 4. Raw data of sample readings and protein concentration calculation of ELISA to measure hIL-13 concentrations in alfalfa transgenic and wild type plants.

Root Line 2	R1	R2	R3	Average	Protein C. (ng/ 100 μg)
E1	0.765	0.797	0.782	0.782	69
E2	0.895	0.879	0.882	0.886	86
E3	0.651	0.638	0.672	0.654	65
Stem Line 2					
E1	0.541	0.559	0.545	0.549	43
E2	0.411	0.437	0.458	0.435	33
E3	0.352	0.396	0.405	0.383	30
Leaf Line 2					
E1	1.119	1.163	1.172	1.151	110
E2	0.908	0.862	0.886	0.885	86
E3	0.741	0.714	0.739	0.731	75
SE Line 2					
E1	1.682	1.701	1.718	1.7	172
E2	2.031	1.985	2.049	2.016	219
E3	1.908	1.914	1.996	1.939	232
Root Line 7					
E1	1,247	1.348	1.316	1.304	127
E2	1.005	1.018	1.041	1.021	102
E3	0.824	0.829	0.817	0.823	87
Stem Line 7					
E1	0.986	1.027	0.923	0.98	91
E2	1.169	1.135	1.193	1.166	119
E3	1.082	0.975	1.061	1.039	115
Leaf Line 7					
E1	1.205	1.124	1.231	1.187	114
E2	1.283	1.289	1.206	1.259	130
E3	1.43	1.328	1.305	1.354	156
SE Line 7					
E1	1.849	1.934	1.966	1.916	196
E2	1.33	1.316	1.384	1.344	140
E3	1.389	1.268	1.442	1.37	158

Root Line 11	R1	R2	R3	Average	Protein C. (ng/ 100 µg)
E1	1.268	1.217	1.156	1.214	117
E2	0.961	0.924	0.922	0.936	92
E3	0.841	0.957	0.925	0.908	98
Stem Line 11					
E1	0.927	1.218	0.845	0.908	83
E2	0.982	0.998	1.007	0.996	99
E3	0.771	0.683	0.645	0.7	71
Leaf Line 11					
E1	1.218	1.061	1.19	1.16	111
E2	0.862	0.827	0.916	0.868	84
E3	0.841	0.873	0.849	0.854	91
SE Line 11					
E1	1.314	1.399	1.438	1.385	137
E2	1.839	1.863	1.835	1.846	199
E3	1.583	1.624	1.664	1.624	191
WT					
E1	0.248	0.225	0.227	0.233	7
E2	0.239	0.213	0.204	0.219	7.6
E3	0.214	0.236	0.208	0.219	8.6

Note: E, experiment; R, repeat; Protein C., protein concentration; SE, somatic embryo; WT, wild type alfalfa.