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**GENE TRANSFER, GENE EXPRESSION, AND
SOMATIC EMBRYOGENESIS IN TWO LEGUME
SPECIES: SOYBEAN AND ALFALFA**

by

LINING TIAN, B.Sc., M.Sc.

**A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfilment of the requirements for the degree of
Doctor of Philosophy**

Department of Biology

Carleton University

Ottawa, Ontario

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**"GENE TRANSFER, GENE EXPRESSION, AND
SOMATIC EMBRYOGENESIS IN TWO LEGUME
SPECIES: SOYBEAN AND ALFALFA"**

submitted by

LINING TIAN, B.Sc., M.Sc.

in partial fulfilment of the requirements

for the degree of Doctor of Philosophy

Chair, Department of Biology

Thesis co-supervisor

Thesis co-supervisor

External Examiner

1995, Carleton University

ABSTRACT

Long-day photoperiod-adapted (LDPA) soybean germplasms were screened for their ability to form somatic embryos. The 20 cultivars screened formed between 1 and 7 embryos per cotyledon with between 18% and 98% of the cotyledons responding. At least one ancestral line, 840-7-3, was prominent in the genetic background of the varieties which showed a high degree of embryogenesis. Differential responses were found for two embryogenesis systems. The development of somatic embryogenesis system for LSPA soybean will provide a base for many pure and applied research studies.

A simple particle bombardment device was developed and characterized. In this system, DNA-coated tungsten particles were accelerated directly by pressurized helium without using consumable materials. The device delivers genes into soybean and alfalfa cells as efficiently as a commercial device does. The operating cost and set up time for bombardment was reduced. The development and characterization of this new device will greatly facilitate the use and distribution of biolistic approach in many laboratories.

Abiotic and biotic factors were investigated and studied for their influence on alfalfa transformation using biolistic approach. The explants and developmental stage of the explants in culture strongly influenced GUS gene expression. Different types of media resulted in substantially different levels of transgene expression. The results suggest that petioles at day 5 in culture and developed somatic embryos are superior explants for development of a system for stable transformation of alfalfa.

Embryogenic suspension cultures of Nattawa were bombarded using the plasmid PBI221.23 carrying HYG and GUS genes. The hygromycin-selected cultures showed GUS gene expression. PCR showed the cultures were positive for the GUS gene. Southern Blot analysis of the cultures indicated that the GUS gene and the HYG gene were incorporated into the plant genome. Somatic embryos developed from these cultures. The transformation system developed will provide potential to transform soybean with agronomically important genes.

ABA was used to induce desiccation tolerance in somatic embryo of soybean to develop a method which may potentially preserve transformed embryogenic lines. A stably transformed soybean embryogenic line showed tolerance to desiccation under RH 43% for at least three days after maturation of embryos. ABA treatment of the embryogenic culture, at all stages of development, did not result in an induction of desiccation tolerance.

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LIST OF ABBREVIATIONS

2,4-D:	2,4 dichlorophenoxyacetic acid.
ABA:	Absciscic acid.
GUS:	<i>uidA</i> gene, coding for β-glucuronidase.
EDTA:	Ethylenediaminetetra-acetic acid.
HYG:	Gene conferring hygromycin resistance.
LDPA:	Long-day-photoperiod-adapted.
MS:	Murashige and Skoog (1962) medium.
NAA:	α-naphthalene acetic acid.
NPTII:	Gene conferring resistance to kanamycin.
PCR:	Polymerase chain reaction.
PCV:	Packed cell volume.
PEG:	Polyethylene glycol.
RH:	Relative humidity.
SDPA:	Short-day-photoperiod-adapted.
X-Gluc:	5-bromo-4-choro-3-indolyl glucuronic acid

GENERAL INTRODUCTION

I. Genetic engineering of plants

For many years, conventional breeding has been used to improve crop qualities, such as disease resistance, herbicide resistance, drought tolerance, and nutrient quality. While this method produces many new cultivars with desirable traits, some disadvantages are also associated with it. Breeding usually results in a wide genetic recombination which, in turn, influences these and other quantitative traits. This approach is also time and labour consuming since several back-cross cycles are usually required before a cultivar with desirable traits can be obtained. Development of recombinant DNA techniques and biotechnologies makes it possible to engineer plants more precisely, specifically, and efficiently. Furthermore, such techniques have broadened the gene pool and allowed transfer of specific genes from one organism to another which would be otherwise sexually incompatible.

When more and more genes of agronomic interest were identified, isolated, characterized, and become available for plant genetic engineering, various gene transfer methods were also developed, such as *Agrobacterium*-mediated transformation (see Weising *et al.*, 1988; Hooykaas, 1989), electroporation (Toriyama *et al.*, 1988; Shimamoto *et al.*, 1989; Sagi *et al.*, 1994; Lause *et al.*, 1994), polyethylene glycol (PEG)-mediated fusion (Haffman *et al.*, 1994), microinjection (Crossway *et al.*, 1986; Reich *et al.*, 1986), silicon carbide fibre-mediated transformation (Kaeppeler *et al.*, 1990; Frame *et al.*, 1994), liposome-mediated gene delivery, virus-mediated DNA delivery, and laser-mediated gene transfer (see Potrykus, 1990, 1991).

Agrobacterium-mediated transformation has been the method most widely used for DNA transfer in to plants. This method depends on the susceptibility of the target plant cells to *Agrobacterium* as well as the regeneration of infected tissues into whole plants. However, the restricted host range of *Agrobacterium* limits the use of this approach in many plants, especially in monocotyledon and legume species.

The biolistic process (also refereed to as biological ballistics, microprojectile bombardment, the gene gun method, the particle acceleration method) is a novel technique to deliver nucleic acid into plant cells. This system was first demonstrated by Klein *et al.* in 1987. Small tungsten microprojectiles (spherical particles) were accelerated by a particle gun to velocities at which these microprojectiles could penetrate intact epidermal cells of *Allium cepa* (onion). The bombarded cells were found to survive the penetration of the microprojectiles. By coating the microprojectiles with DNA, foreign genes were introduced into plant cells and the activities of the foreign gene were detected. The biolistic process does not need any intermediate gene delivery agent and is able to introduce any of a wide range of foreign genes directly into living cells of any plants species. Therefore, this method circumvents the limitations of using *Agrobacterium*-mediated gene transfer approach, and thus, offers the opportunity for gene transfer into a variety of plants.

Following the initial report of delivery of genes into plant cells, the biolistic process has been used to transfer DNA into a wide variety of plant species. In addition to delivering genes into nuclei, this approach has also been demonstrated to be useful for transformation of organelles, such as chloroplasts (Daniell *et al.*, 1990, 1991) and

mitochondria (Fox *et al.*, 1988). Stable transformation has also been reported in a number of species, including maize (Klein *et al.*, 1988, 1989, 1990; Gordon-Kamm *et al.*, 1991; Dennehey *et al.*, 1994), tobacco (Tomes *et al.*, 1990), cotton (Finer and McMullen, 1990), papaya (Fitch *et al.*, 1990), rice (Christou *et al.*, 1992; Cao *et al.*, 1992; Li *et al.*, 1993), yellow-poplar (Wilde *et al.*, 1992), oat (Sommers *et al.*, 1992), wheat (Vasil *et al.*, 1992; Nehra *et al.*, 1994; Becker *et al.*, 1994), turfgrass (Zhong *et al.*, 1993), bean (Russell *et al.*, 1993), peanut (Brar *et al.*, 1994), and barley (Ritala *et al.*, 1993, 1994). This technique has also been used in transformation of a diverse range of organisms, including bacteria, algae, yeast, filamentous fungi, and animals.

II. Genetic engineering of soybean

A) Research background

Soybean [*Glycine max* (L.) Merrill] is the most important legume crop in the world, being an important source of vegetable oil and protein. It also provides many raw materials for the chemical industry. Around 50 million hectares are planted annually and the world production of soybean is around 105 million tons. Soybean is ranked number one in the world for vegetable oil production (Hymowitz, 1988).

Current breeding lines and cultivars of soybean were developed through classical breeding approaches. However, present soybean germplasm variability is extremely narrow and the majority of the soybean cultivars in use are derived from very few

parental lines (Cristou *et al.*, 1990). As a result, development of new traits via conventional breeding methods have been restricted.

As an alternative approach, some effort has been made towards the improvement of soybean cultivars via genetic engineering techniques. Soybean can be infected by *Agrobacterium*, but it is relatively resistant to the infection compared to many other crop plants (Hinchee *et al.*, 1988). Wang and co-workers (1983) reported that only 2.5% of nearly 1000 soybean cultivars were susceptible to tumour induction by *Agrobacterium*. Previously, foreign genes were transferred only by oncogenic *Agrobacterium* Ti vectors (Facciotti *et al.*, 1985; Owens and Cress, 1985; Pedersen *et al.*, 1983; Hood *et al.*, 1986; Jiang *et al.*, 1986; Kudirka *et al.*, 1986; Baldes *et al.*, 1987).

The first demonstration of soybean transformation was by Hinchee *et al.* in 1988, using *Agrobacterium*. Cotyledon explants of the variety Peking were inoculated with *Agrobacterium* and plants recovered under kanamycin selection conditions expressed the introduced NPTII and GUS genes. Subsequently, several laboratories also reported transformation of soybean using *Agrobacterium* (Parrott *et al.*, 1989b; Zhou and Atherly, 1990). Nevertheless, all the transformations succeeded in the same genotype, Peking, and no progeny were generated from the transformed plants. To date, Peking, which is not a commercially important cultivar, is the only soybean cultivar successfully transformed via *Agrobacterium*. Generally, soybean is not considered a good host for *Agrobacterium* infection.

Liu *et al.*, (1990) demonstrated transformation of soybean by the microinjection of ovules. However, the report did not provide convincing evidence for stable

transformation (Christou, 1994).

For the past several years, the biolistic approach has been used to transform soybean cultivars. Bombardment of the meristems of embryogenic axes and regeneration of the bombarded tissues can lead to transformation (McCabe *et al.*, 1988). By introducing DNA-coated gold microprojectiles with a particle gun (electric charger) into meristems of immature soybean seeds, approximately 2% of shoots derived from these meristems via organogenesis were chimeric for the expression of the introduced gene. Nonchimeric second-generation plants, which were fertile, were subsequently obtained from the transformed plants. Although transformation of soybean via this system is possible, some disadvantages are associated with it. The most salient one is that the regenerated plants are usually chimeric for the foreign gene. The reason for this is shoots regenerated via organogenesis are multicellular in origin. It is unlikely that all the cells from which one shoot is derived were transformed originally by the foreign gene. Large numbers of shoots must be regenerated and recovered in the greenhouse and only in the F1 generation produced from these shoots can plant selection for nonchimeric plants begin. In addition, no selection procedure was applied in the system (McCabe *et al.*, 1988) and thousands of plants recovered from bombarded meristems needed to be assayed. Although this approach has produced many transformed plants and appeared to be genotype independent, it was obviously very time and labour consuming. To date, this system has been successful in only one laboratory (McCabe *et al.* 1988; Christou *et al.*, 1987, 1988, 1989a, 1990; Yang and Christou 1990), and attempts did not succeed in other laboratories (Sato *et al.*, 1993).

The use of somatic embryo culture is a possible alternative approach. Somatic embryos usually originate from the progeny of a single cell. Therefore, bombardment of embryogenic cultures is likely to result in non-chimeric plants. Successful recovery of non-chimeric transformed plants via somatic embryogenesis was demonstrated in rice (Li *et al.*, 1993) and turfgrass (Zhong *et al.*, 1993). The successful establishment of an efficient somatic embryogenesis system is the prerequisite for this transformation approach. Somatic embryogenesis in soybean was developed relatively recently, in the mid to late 1980's. By bombarding an embryogenic suspension culture (cv. Fayette), Finer and McMullen (1991) demonstrated transformation and recovery of a non-chimeric soybean plant.

B) Problems

Somatic embryogenesis and related gene transfer approaches are strongly dependent on lines or germplasms of the species. In 1920, Garner and Allard discovered photoperiodism. Soybean (cv Biloxi) was one of the plants used by them in the experiment (see Street and Opik, 1984). Soybean was found to have a requirement of long dark periods for flowering. To date, all studies on soybean somatic embryogenesis and transformation via the biolistic approach were those cultivated at low latitude. At high latitudes, ordinary soybean will not flower until late August or September and then there may not be enough time for seeds to mature before they are frozen. As a result, high-latitude-adapted soybean was developed. These soybean

germplasms and cultivars were developed from sources that are different from those adapted to lower latitude areas. They are unique for two properties; namely, daylight insensitivity, or adaption to long-day photoperiods. These germplasms and cultivars are agronomically important and widely cultivated in the world. A somatic embryogenesis system and efficient transformation technique still needs to be fully developed for this valuable germplasm.

III. Genetic engineering of alfalfa

A) *Research background*

Alfalfa (*Medicago sativa* L.) (also called lucerne) is the most important forage legume in the world. This perennial plant is mainly cultivated in Europe and North America, providing a major source of protein for dairy and beef production.

Compared to soybean and other legume species, alfalfa tissue culture is at a more developed level. Alfalfa was one of the first major crops in which plants were regenerated from tissue culture (Saunders and Bingham, 1972; Bingham *et al.*, 1975). Since that time, alfalfa tissue culture and regeneration have been improved dramatically and plants can be regularly regenerated from a variety of explants (Johnson *et al.*, 1981; Meijer and Brown, 1985; Lupotto, 1986; Dijak and Brown, 1987; Bingham *et al.*, 1988; Denchev *et al.*, 1993; Finstad *et al.*, 1993; Lai and McKersie, 1993, 1994). Now, alfalfa *in vitro*-regeneration systems have been well developed, which provides many

basic and applied research opportunities.

Transformation in this legume species also appears to be at a more advanced level than many legumes. Alfalfa shows more receptivity to *Agrobacterium*, compared to soybean. Both *A. tumefaciens* and *A. rhizogenes* have been reported to be effective in transferring genes into alfalfa. Various explants can be used for *Agrobacterium* infection and *Agrobacterium*-mediated transformation of alfalfa has been reported by many laboratories (Deak *et al.*, 1986; Shahin *et al.*, 1986; Sukhapinda *et al.*, 1987; Spano *et al.*, 1987; Chabaud *et al.*, 1988; Pezzotti *et al.*, 1991; Schroeder *et al.*, 1991; McKersie *et al.* 1993; Desgagnes *et al.*, 1995; Du *et al.*, 1994). Hill *et al.* (1991) recovered over one hundred transgenic plants which expressed the coat protein of alfalfa mosaic virus (AMV). The plants were resistant to AMV and did not develop systemic infection. Transgenic alfalfa plants resistant to herbicide were also recovered and evaluated in field trials (D'Halluin *et al.*, 1990).

B) Problems

Despite the fact that alfalfa is more receptive to *Agrobacterium*, transformation of alfalfa via *Agrobacterium* still appears to be strongly genotype dependent. Du *et al.* (1994) tested several genotypes and only one genotype was transformed via *Agrobacterium*. Before a successful transformation system can be developed, an extensive search for a suitable *Agrobacterium* strain/vector and plant genotype/explant is needed (Desgagnes *et al.*, 1995). This approach is time and labour consuming. Thus,

the development of other transformation methods is still needed in order to improve many varieties of alfalfa which are recalcitrant to *Agrobacterium* infection.

Somatic embryogenesis in alfalfa has been well developed and characterized (see review Bingham *et al.*, 1988). Nevertheless, an efficient transformation system of alfalfa based on somatic embryogenesis via microprojectile bombardment has not been developed yet. In an effort to achieve stable transformation, Pereira and Erickson (1995) bombarded over 2000 alfalfa petioles and stem explants and obtained only one single transformant which later developed into 7 plants, in which, only a piece of the foreign DNA was found. Obviously, the protocol used was far from optimal. Various factors and conditions necessary for establishment of an efficient transformation system for alfalfa still need to be characterized and evaluated.

IV. Research hypotheses and objectives

A. Somatic embryogenesis of LDPA soybean

Development of a somatic embryogenesis system will be of benefit to many basic and applied research programs. In addition, a reliable and efficient system for *in vitro* regeneration via embryogenesis is the prerequisite for establishment of transformation via microprojectile bombardment. Such a system for LDPD soybean has not been developed and systematically evaluated yet. Establishment of a somatic embryogenesis system for LDPA soybean will provide a base for various kinds of research in this germplasm and

was therefore the first goal of this project. A previous study (Ranch *et al.*, 1985) has suggested that high-latitude-adapted soybean be more responsive to somatic embryogenesis. The following hypothesis will be tested:

Hypothesis

The capacity of somatic embryogenesis of LDPA soybean is different from ordinary soybean. LDPA soybean is more responsive to somatic embryogenesis.

Objective

Establishment of a somatic embryogenesis system and characterization of the system for LDPA soybean.

B. Improvement of the biolistic bombardment device

Commercial bombardment devices are expensive and operation is complicated. Characterization, modification, and improvement of bombardment device will facilitate the use of biolistic technology. A simple particle bombardment device, based on Particle Inflow Gun (Finer *et al.*, 1992), was developed in Dr. D. C. W. Brown's laboratory, Plant Research Centre, Agriculture Canada (Brown *et al.*, 1994). In this system, DNA-coated tungsten particles were accelerated directly by pressurized helium without using

consumable materials. This device is inexpensive to build and entails a relatively low operating cost. The set up time for each bombardment is also reduced. Chapter 2 reports the characterization and optimization of this new particle bombardment device for gene delivery in alfalfa and soybean cells.

Finer *et al.* (1992) reported that Particle Inflow Gun delivered gene into plant cells efficiently. However, the efficiency for gene delivery using PIG was not compared to a commercial gun. The new device developed in Dr. Brown's laboratory has incorporated an electronic system and controls the operation more precisely and efficiently. Following hypothesis will be tested:

Hypothesis

The new Particle Bombardment Device will deliver DNA, after optimization, into alfalfa and soybean cells as efficiently as a commercial device does.

Objective

Characterization and optimization of the new Particle Bombardment Device.

C. Transformation of alfalfa using microprojectile bombardment

Development of a biolistic-mediated transformation system will minimize the

limitation of the *Agrobacterium*-mediated transformation approach. This section of the project investigated the potential of development of an efficient transformation system in alfalfa by microprojectile bombardment using a somatic embryogenesis system. Various factors and conditions for the transformation of alfalfa via microprojectile bombardment were investigated and evaluated. The genotype independence of the microprojectile bombardment approach was investigated.

Previous studies have demonstrated that the stage of *in vitro* culture affects gene expression (Karthi et al., 1989). Studies also showed that plants, which were poorly responsive to *Agrobacterium* infection, could be transformed by biolistic approach (Ritala et al., 1994; Nehra et al., 1994). Studies were carried out to test the following hypotheses.

Hypothesis

- i). Development stages of the somatic embryogenesis in alfalfa affect transformation following microprojectile bombardment.
- ii). Microprojectile bombardment will circumvent, or at least, minimize the host-range limitation of *Agrobacterium*-mediated approach in alfalfa transformation.

Objective:

Evaluation of various biotic and abiotic conditions for the potential of development of an efficient transformation system by microprojectile bombardment in alfalfa.

D. Development of a biolistic transformation technology for LDPA soybean germplasm types.

To date, all the studies on soybean transformation were in the genotypes and germplasms adapted to short-day-photoperiods and soybean genetic engineering currently remains inefficient and definitely not routine. Development of a transformation technology will provide a way to genetically modify LDPA soybean cultivars by transferring different kinds of genes into the plant. A previous study has demonstrated that after bombardment of somatic embryos non-chimeric-transformed plants could be recovered (Finer and McMullen 1991). The studies will test following hypothesis and achieve following objectives.

Hypothesis:

LDPA soybean is able to be transformed via microprojectile bombardment using a somatic embryogenesis system and plants recovered would be non-chimeric for foreign gene.

Objective:

- i). Evaluation of the effectiveness of using a biolistic process for gene transfer in LDPA soybean.
- ii). Development of a biolistic transformation technology for LDPA soybean germplasm types.

E. Maturation of somatic embryos and preservation of transformed soybean lines by artificial seed technology.

Chapter 5 presents the data on the study and characterization of embryo maturation, ABA treatment, and desiccation tolerance in a transformed soybean embryogenic line. Preservation of transformed cell lines will facilitate the use of genetic engineering. To date, there is no study on this subject. Somatic embryos, such as in alfalfa (McKersie *et al.*, 1989; Senaratna *et al.*, 1989, 1990; Brown 1994), and carrot (Iida *et al.*, 1992), after certain treatments, e.g. ABA, can be preserved in dry conditions for long times without losing viability.

Hypothesis:

ABA treatment can promote embryo maturation and induce desiccation tolerance

in a transformed soybean line.

Objective:

Development of a method using artificial seed technology to preserve transformed soybean lines.

CHAPTER 1

***IN VITRO* RESPONSE AND PEDIGREE ANALYSIS FOR SOMATIC EMBRYOGENESIS OF LONG-DAY -PHOTOPERIOD-ADAPTED SOYBEAN**

INTRODUCTION

For some time, soybean was conspicuous among major crops because it lacked an *in vitro* regeneration system. This deficiency impeded application of many biotechnologies to improve the plant. Soybean was one of first species from which a serially-propagated cell suspension was generated (Gamborg *et al.*, 1968) and one of the first species from which protoplasts were isolated (Schenk and Hildebrandt, 1969). Cell proliferation from protoplasts of soybean was first demonstrated by Kao *et al.* (1970). However, difficulty was met in the effort to regenerate soybean plants from these unorganized cell cultures in spite of numerous technical approaches. Soybean cell and tissues were recalcitrant to every known regeneration protocol.

The evidence of the early stages of somatic embryogenesis was first presented by Beversdorf and Bingham (1977), but none of the embryos developed into plants. Subsequently, Phillips and Collins (1981) and Gamborg *et al.* (1983) presented similar evidence for embryogenesis in soybean suspension cultures. In all cases, the structures observed superficially resembled somatic embryos, however, they were not competent to develop since whole plants could not be obtained. Christianson *et al.* (1983) produced a morphogenetically competent culture from the embryogenic axes of immature soybean embryos, and following that, Lippmann and Lippmann (1984) reported somatic embryogenesis from immature cotyledons. Again, whole plants were not recovered.

Regeneration of soybean via somatic embryogenesis was not successful until the mid 1980's. Ranch *et al.* (1985) and Lazzeri *et al.* (1985) first demonstrated plant

regeneration from somatic embryos. Somatic embryos were induced from cotyledons of immature zygotic embryos with the use of auxins 2,4-D or NAA at a relative high concentration. Somatic embryos developed were picked up and transferred to media containing lower concentrations of growth regulators for maturation and germination. The actual induction of somatic embryos from immature cotyledons of soybean appeared to be relatively flexible and it occurred in a range of culture conditions (Lazzeri *et al.*, 1985). Explant choice, however, proved to be the important missing key in the successful recovery of plants. To date, specifically-staged cotyledons of immature embryos are the most useful explant source from which somatic embryos can develop and whole plants can be recovered.

Subsequent to the initial reports of recovery of plants from somatic embryos, various culture conditions have been investigated and characterized in detail to optimize the regeneration system (Barwale *et al.*, 1986; Hammatt and Davey, 1987; Komatsuda and Ohyama, 1988; Christou and Yang, 1989; Parrott *et al.* 1989; Buchheim *et al.*, 1989; Komatsuda and Ko, 1990; Komatsuda, 1990, 1992; Shoemaker *et al.*, 1991; Wright *et al.*, 1991; Bailey *et al.*, 1993a). With the optimized conditions, induction of somatic embryos and recovery of plants has become routine in many laboratories. Nevertheless, the induction efficiency was generally low, ranging from less than one to 2-3 embryos per cotyledon explant, depending on the genotypes and culture protocols. Of these, only a portion exhibited the normal morphology which usually led to germination and conversion into plants. This greatly limited the practical use of somatic embryogenesis. Finer and Nagasawa (1988) made great progress in soybean

embryogenesis with respect to this limitation. Somatic embryos and embryogenic tissue developed from immature cotyledons were transferred into a liquid medium for proliferation. Upon transfer the suspension-cultured tissues to embryo-development medium, numerous somatic embryos developed. Development of the embryogenic suspension culture provided a new route for the use of soybean somatic embryos.

Although there have been numerous studies on soybean somatic embryogenesis, somatic embryogenesis of soybean varieties adapted to long-day photoperiods has not been investigated and characterized. Establishment of a somatic embryogenesis system is the prerequisite for development of a gene transfer technique for this germplasm. Such a system will also provide a base to apply many biotechnologies in these soybean germplasms. This project focused on the investigation of somatic embryogenesis of soybean germplasms which have been adapted to a high latitude of 45° North.

MATERIALS AND METHODS

I. Donor plants

Soybean seeds were obtained from the soybean breeder Dr. H. Voldeng, Plant Research Centre, Agriculture Canada, Ottawa. Seeds were germinated in vermiculite. Germinated seeds were transferred into 15 cm x 15 cm fiber pots with a 1:3:1 soil:peat:sand potting mix and grown in a cycle of 20-hr light at 27 °C and 4-hr darkness at 22 °C in a growth chamber. Fifty to 60 days after germination the plants were transferred to a growth chamber with a 12-hr light at 25 °C and 20 °C in darkness for flower induction with a light intensity of 200 $\mu\text{mol m}^{-2}\text{s}^{-1}$ using Sylvania "Cool White" fluorescent tubes.

II. The standard culture protocol

Soybean pods, harvested two to three weeks post-flowering, were surface sterilized with 70% ethyl alcohol for 2 min followed by treatment for 20 min in a saturated 7% calcium hypochlorite solution and 3 washes of sterile distilled water. Immature seeds of 4.5-5.5 mm in length were aseptically removed from the pods and excised individual cotyledons were transferred to a medium (MSB) containing MS salts (Murashige and Skoog, 1962), B5 vitamins (Gamborg *et al.*, 1968), 180 μM 2,4-D, 43.5 mM sucrose and 0.8% Difco Bacto agar (pH 5.8). The *in vitro* protocol was based on

that of Ranch *et al.* (1985) and Lazzeri *et al.* (1985). Ten cotyledons were normally cultured in each Petri dish (60 x 20 mm) containing 25 ml medium. The dish was sealed with three layers of Parafilm (American National Can[™]). The media were sterilized by autoclaving at 121 °C for 18 min. The cotyledons were placed abaxial side down and the culture was maintained at 25°C under a 16-hr photoperiod with a light intensity of 25 $\mu\text{mol m}^{-2}\text{s}^{-1}$; Sylvania "Cool White" fluorescent tubes. After 14 days on MSB medium, the cotyledons were transferred and maintained on the same medium, lacking growth regulators. After 16 days on growth regulator-free medium, somatic embryogenesis was evaluated. A somatic embryo was identified as an individual and separate embryo-like structure which could be potentially picked up by a forceps.

III. Optimization of *in vitro* culture conditions for somatic embryogenesis

i) 2,4-D concentration

Somatic embryogenesis was first tested with different concentrations of 2,4-D. Two lines, Nattoson and X2558-13-3-1 (X2558) were used in the study. The cotyledons were cultured for 14 days in standard MSB medium which contained concentrations of 2,4-D (Sigma) at 112, 180, 248, 315, and 450 μM . The range of 2,4-D concentrations was selected based on published protocols (e.g. Parrott *et al.* 1989; Bailey *et al.*, 1993a). After 14 days, the cotyledons were transferred and maintained on the same medium, lacking growth regulators, for an additional 16 days. Somatic embryos were counted 30

days after culture initiation as a measure of the function of the growth regulator treatment.

ii) Induction time

The induction of somatic embryogenesis with 2,4-D was also tested by exposing the cotyledons to 2,4-D for different lengths of time. Cotyledons of Nattoson and X2558-13-3-1 were induced for embryogenesis with 180 μ M 2,4-D in standard MSB medium for 1, 3, 7, 10 and 14 days. After induction, the cotyledons were transferred and maintained on the same medium, lacking growth regulators, for up to 30 days. The number of somatic embryos formed per cotyledon were counted 30 days after culture initiation.

IV. Genotype screening for highly embryogenic lines

Twenty soybean lines, which are all adapted to high-latitude, were tested for somatic embryogenesis. A minimum of 67 cotyledons were tested from each genotype and, generally, more than 100 cotyledons were used. Immature cotyledons of 4.5-5.5 mm in size were cultured in MSB medium. After 14 days, the cotyledons were transferred and maintained on the same medium, lacking growth regulators. Thirty days after introduction into culture, the number of somatic embryos developed on MSB medium was recorded and embryogenesis was evaluated for these lines. Four parameters

were used to assess the embryogenesis:

- 1) percent surviving cotyledons,
- 2) frequency response (percent cotyledon responding),
- 3) mean embryo number per surviving cotyledon, and
- 4) mean embryo number per responding cotyledon.

Because the frequency distribution of the number of somatic embryos formed on a cotyledon explant tended to a Poisson distribution, for analysis, data were transformed by a square root transformation and subjected to a one-way ANOVA. Data expressing embryos per surviving cotyledon were subjected to a Duncans Multiple Comparison Test.

V. Suspension culture for somatic embryogenesis

Seven lines; namely, X2683-47-1-1 (X2683), X2558-13-3-1 (X2558), 840-7-3 (840), Maple Arrow (Arrow), Canatto, TNS, and Nattawa, which represented a high, medium, and low capacity to form somatic embryos in the induction medium (see Table 1.3) were tested for their embryogenesis ability in suspension culture. Thirty to forty mg of somatic embryos with early-staged-embryogenic callus, which had developed on the cotyledons (about 40 to 50 days after culture initiation), was transferred into 40 ml of suspension culture medium in a 125 ml flask. The suspension culture medium consisted of MS salts, B5 vitamins, 22.5 μM 2,4-D, 6% sucrose, and 15 mM glutamine (pH 5.7) (Ms medium). The flasks were closed with a foam plug and agitated at 140 rpm at low light ($15 \mu\text{mol m}^{-2}\text{s}^{-1}$) under a 16-h photoperiod. At least three flasks were

initiated for each genotype. Suspension cultures were subcultured every month. Embryogenic suspension cultures proliferated as spherical clumps of globular embryos. Using the formula for calculating the volume of a sphere, $V = 4/3(\pi r^3)$, an approximation of packed cell volume (PCV) was determined by counting the number and size (diameter) of embryogenic clumps. The growth rate, measured as packed cell volume (Finer and Nagasawa, 1988) for each flask, was thus recorded. For subculture, two embryogenic tissues about 4 mm in diameter were placed into fresh medium. Meanwhile, some embryogenic clumps were transferred and maintained on a MSBM medium for embryo development. MSBM medium consisted of MS salts (Murashige and Skoog, 1962), B5 vitamins (Gamborg *et al.*, 1968), 6% maltose and 0.25% Gelrite (pH5.8). Somatic embryogenesis in suspension culture was determined by observing the embryo development from embryogenic clumps on MSBM medium one month afterwards.

VI. Embryo germination and conversion

After one month on MSBM medium, embryos were subjected to two different treatments; that is, either direct germination or maturation before germination. For direct germination, somatic embryos were separated from each other and transferred to a sterile empty dish which was then sealed with two layers of Parafilm. The dish was placed in the same conditions as for embryo induction and development. The embryos were partially desiccated in this condition for 5-7 days and then transferred back to fresh MSBM medium for germination. For maturation, separated embryos were transferred

to MBO medium and maintained for one month. MBO medium containing MS salts, B5 vitamins, 4% sucrose, and 0.25% Gelrite at pH 5.8 for maturation. After one month on MBO medium, the embryos were desiccated as before. Desiccated embryos were returned to fresh MBO medium for germination. Germination was defined as root development and shoot elongation.

A diagram showing the general protocol of soybean tissue culture can be seen in Fig. 1.1.

Fig. 1.1. Diagram showing the protocol of somatic embryogenesis in soybean.

- 1 Cotyledons in MSB medium for embryo induction.**
- 2 Suspension culture.**
- 3 Embryo development.**
- 4 Embryo maturation.**
- 5 Partial desiccation.**
- 6 Embryo germination.**
- 7 Plant recovery.**

RESULTS

After 15-20 days on MSB induction medium, somatic embryos started to form on the adaxial surface and margins of cotyledons. The morphology of somatic embryos resembled globular-staged zygotic embryos. Somatic embryos were translucent and pale yellow. The embryos never germinated in induction medium.

I. Optimization of *in vitro* culture for somatic embryogenesis

i) 2,4-D concentration

Preliminary experiments showed that Nattason and X2558-13-3-1 (X2558) gave a relatively higher number of somatic embryos and they were subsequently used in this experiment. The data of somatic embryos production in these genotypes were more likely to assume a normal distribution and therefore could be tested statistically (Komatsuda, 1992). Investigation of these two genotypes showed that a wide range of 2,4-D concentration from 112 to 450 μM all exhibited induction of somatic embryogenesis (Table 1.1a). Explants cultured at 180 μM and 248 μM 2,4-D resulted in the highest number of embryos for both genotypes. Higher 2,4-D concentration, i.e. at 315 and 450 μM , showed decreased induction efficiency.

Table 1.1a. Effect of 2,4-D concentration on somatic embryogenesis of two soybean genotypes of Nattason and X2558. Cotyledons were cultured on MSB medium for 14 days and then transferred and maintained on the same medium, lacking growth regulators. After 16 days on growth regulator-free medium, somatic embryogenesis was evaluated. The values represent the mean $\pm$ standard error of the mean. At least 25 (maximum 60) cotyledons were used for each 2,4-D concentration for each genotype.

Genotype	Mean no. of embryos/cotyledon $\pm$ se				
	2,4-D concentration (μ M)				
	112	180	248	315	450
Nattason	4.37 $\pm$ 0.44	5.90 $\pm$ 0.59	6.20 $\pm$ 0.60	4.51 $\pm$ 0.75	3.92 $\pm$ 0.73
X2558	2.82 $\pm$ 0.51	5.13 $\pm$ 0.72	7.23 $\pm$ 0.94	6.27 $\pm$ 0.57	4.31 $\pm$ 0.61

Table 1.1b. Analysis of variance of effect of 2,4-D concentration on somatic embryogenesis of two soybean genotypes of Nattason and X2558 (See Table 1.1a for experimental procedure and design).

Source	DF	Sum of square	Mean square	F	P
Genotype	1	1.61	1.61	1.42	0.2337
2,4-D	4	33.24	8.31	7.32	0.0001
Geno. * 2,4-D	4	16.39	4.10	3.61	0.0066
Error	411	466.42	1.13		

Table 1.2a. Effect of induction time, using 2,4-D, on somatic embryogenesis of two soybean genotypes, Nattason and X2558. Cotyledons were cultured with 180 μ M 2,4-D in standard MSB medium for 1, 3, 7, 10 and 14 days. After induction, the cotyledons were transferred and maintained on the same medium, lacking growth regulators, for up to 30 days. The number of somatic embryos formed per cotyledon were counted after 30 days. The values represent the mean $\pm$ standard error of the mean. At least 30 (maximum 50) cotyledons were used for each induction time for each genotype.

Genotype	Mean no. of embryos/cotyledon $\pm$ se				
	Time in 2,4-D medium (day)				
	1	3	7	10	14
Nattason	1.04 $\pm$ 0.19	2.28 $\pm$ 0.29	4.78 $\pm$ 0.42	7.85 $\pm$ 0.67	5.98 $\pm$ 1.01
X2558	1.37 $\pm$ 0.27	3.27 $\pm$ 0.46	3.43 $\pm$ 0.51	4.70 $\pm$ 0.54	4.61 $\pm$ 0.52

Table 1.2b. Analysis of variance of effect of induction time, using 2,4-D, on somatic embryogenesis of two soybean genotypes, Nattason and X2558 (See Table 1.2a for experimental procedure and design).

Source	DF	Sum of square	Mean square	F	P
Genotype	1	85.57	85.57	7.38	0.0069
Time	4	1104.38	276.09	23.82	0.0001
Geno. * Time	4	189.42	47.36	4.09	0.0030
Error	380	4404.10	11.59		

Two-factor-analysis of variance (Table 1.1b) indicates that there is a significant difference for somatic embryogenesis induced with different concentrations of 2,4-D tested. There is an interaction between genotype and 2,4-D concentration.

ii) Induction time

One-day exposure to 2,4-D was sufficient to induce embryogenesis (Table 1.2a). Continued exposure to 2,4-D increased embryo yield. An exposure of 10 days to 2,4-D gave rise to the highest numbers of embryos for both genotypes. However, exposure beyond 10 days did not show an increased effect and the embryo number appeared to decrease.

Analysis of variance (Table 1.2b) indicates that there is a significant difference for embryo formation when the cotyledons were exposed to 2,4-D for different periods of time. The difference between genotypes with respect to induction time is also significantly different. However, there is an interaction between genotype and induction time for embryogenesis.

II. Genotype screening for embryogenesis and pedigree analysis

All the lines tested showed some capacity to form somatic embryos (defined as response) (Table 1.3). But a wide range of response was observed among the genotypes. Responses of about 6 embryos per cotyledon for X2683-47-1-1 to less than one embryo

per cotyledon for TNS and Nattawa were documented. The difference of average number of embryos per cotyledon between the best line (x2683-47-1-1) and the poorest line (TNS) was a 20-fold difference. Fourteen of the 20 genotypes responded at 70-100% frequency. In addition, a very high frequency (98-100%) of cotyledons survived the introduction into *in vitro* culture (survival is defined as cells of the cotyledons having divided and formed callus). There was a strong correlation ($r=0.70$, $P<0.01$, $n=20$) between the percent of responding cotyledons for somatic embryogenesis and the number of somatic embryos per responding cotyledon. One-way variance analysis indicated that the capacity for somatic embryogenesis is significantly different among the genotypes ($P<0.01$).

Table 1.3. Regeneration capacity of 20 soybean genotypes. Genotypes are ranked by frequency of somatic embryos per surviving cotyledon.

Genotype	Embryos/ surviving cotyledon (mean $\pm$ se)	Embryos/ responding cotyledon (mean $\pm$ se)	Cotyledons responding (percent)	Explanted cotyledons (number)	Surviving cotyledons (percent)
X2683-47-1-1	5.88 $\pm$ 0.52 a*	6.85 $\pm$ 0.57	80.00	120	100.00
Nattason	5.86 $\pm$ 0.46 a	6.06 $\pm$ 0.45	96.67	90	100.00
X2558-13-3-1	4.85 $\pm$ 0.46 ab	4.97 $\pm$ 0.47	97.50	80	100.00
Hei Hei 3	4.84 $\pm$ 0.41 ab	5.74 $\pm$ 0.42	84.26	109	99.08
840-7-3	4.56 $\pm$ 0.39 b	5.28 $\pm$ 0.40	86.54	106	98.11
Maple Arrow	4.55 $\pm$ 0.85 bc	7.40 $\pm$ 1.15	62.69	67	100.00
Maple Donovan	4.52 $\pm$ 0.41 bc	5.50 $\pm$ 0.45	82.20	120	98.33
Altona	4.51 $\pm$ 0.27 bc	5.50 $\pm$ 0.29	82.25	260	98.85
Ac Bravor	3.93 $\pm$ 0.28 b-d	5.17 $\pm$ 0.31	76.02	250	78.40
Canatto	3.86 $\pm$ 0.35 b-e	5.29 $\pm$ 0.37	73.00	100	100.00
Acifeng 25	3.86 $\pm$ 0.41 b-e	5.38 $\pm$ 0.48	72.00	127	98.42
Maple Amber	3.84 $\pm$ 0.47 b-e	5.00 $\pm$ 0.54	76.60	105	89.52
Maple Glen	3.84 $\pm$ 0.45 b-e	5.38 $\pm$ 0.53	70.70	106	93.40
OT91-3	3.70 $\pm$ 0.40 b-e	4.44 $\pm$ 0.43	83.33	90	100.00
Early Hakucillo	3.33 $\pm$ 0.29 c-e	4.45 $\pm$ 0.32	74.82	140	99.29
OT89-16	2.91 $\pm$ 0.52 de	5.08 $\pm$ 0.82	57.45	110	100.00
Hei Long	2.90 $\pm$ 0.30 ef	4.29 $\pm$ 0.37	67.69	149	99.33
Harosoy 63	1.84 $\pm$ 0.18 f	3.79 $\pm$ 0.29	48.62	290	100.00
Nattawa	0.60 $\pm$ 0.18 g	2.50 $\pm$ 0.61	24.00	100	100.00
TNS	0.29 $\pm$ 0.06 g	1.57 $\pm$ 0.21	18.50	200	100.00

* Means presented in column 2 & 3 were from untransformed data. Means followed by different letters were found to be significantly different using the Duncan Multiple Comparison Test ($\alpha=0.05$) in which the data were transformed by a square root transformation

Four ancestral lines, 840-7-3, A. K. Harrow, Mandarin, and Altona appeared to be prominent in the genetic background of the best responding lines (Table 1.4).

III. Somatic embryogenesis in suspension culture

The embryogenic tissues of seven genotypes were transferred into liquid medium for establishment of suspension cultures. After one month in suspension culture, embryogenic tissues were selected and separated from nonembryogenic tissues for the use in the next subculture, as described by Finer and Nagasawa (1988). Several subculture periods (i.e. 4-6 months) were needed before an embryogenic suspension culture could be established. Once a highly embryogenic suspension culture was established, such cultures could be maintained for long periods, up to several years by monthly subculture. An embryogenic clump consisted of many globular-staged somatic embryos and such embryos never developed into cotyledonary-staged embryos in suspension culture.

Differential responses were observed for these genotypes. Genotypes, 840-7-3, Maple Arrow, and X2683-47-1-1, which showed higher capacities to form somatic embryos in solid induction medium (Fig. 1-2), did not give a high response in the suspension cultures, with respect to growth rate and the number of the embryos developed on MSBM (Fig. 1.2, 1.3). TNS and Nattawa, which ranked the lowest and second lowest for the capacity of somatic embryogenesis, showed a dramatic increased response for somatic embryogenesis in suspension culture. A mean of 53.8 ± 2.2 embryos were observed from a 4-mm single-embryogenic clump for cv. Nattawa. The

growth of X2683-47-1-1, Maple Arrow, and 840-7-3 was poor and slow, therefore, some of the cultures were unable to be maintained for a long time. Photos showing somatic embryo induction medium and in suspension culture are illustrated in Fig. 1.4, 1.5.

IV. Embryo development, germination and conversion.

The globular-staged embryos in an embryogenic clump were about 1 mm in size. Upon transfer to MSBM medium, embryos started to develop from embryogenic clumps. After two weeks on MSBM medium, the embryos developed into the cotyledonary stage with a size of about 3-4 mm in length. The size of embryos continued to increase and by one month the embryos were 5-7 mm in size. Embryos taken directly from MSBM medium without maturation show poor germination (Table 1.5). Embryos were also transferred onto MBO medium for maturation. The embryos did not show a great change in morphology and size on MBO medium. The embryos matured on MBO were subjected to partial desiccation in an empty Petri dish in which the relative humidity was lower than that in a dish containing medium. After partial desiccation, embryos appeared yellow/white in colour and started to rehydrate after transfer onto fresh MBO medium (Fig. 1.6). The embryos started to germinate one to two weeks after being placed in MBO medium. Matured embryos showed a much higher germination frequency (80%). However, such frequency of germination was achieved only after the desiccation treatment. Germination was characterized by root development and shoot elongation. Conversion was defined as development of primary roots, greening of the cotyledons and

hypocotyl, and formation of the first set of pubescent leaves. Germinated embryos converted into plantlets and were fertile.

Fig. 1.2. Somatic embryogenesis of seven soybean genotypes, X2683, x2558, 840-7-3 (840), Maple Arrow (Arrow), Canatto, Nattawa, TNS, from immature cotyledons in solid induction medium and in suspension culture.

Graph A Embryogenesis from immature cotyledons in solid induction (Mean number of embryos per cotyledon $\pm$ standard error). At least 67 cotyledons were used for each genotype

Graph B Embryogenesis in suspension culture (Mean number of embryos per embryogenic clump $\pm$ standard error). At least 5 embryogenic clumps were used for each genotype

Fig. 1.3. Growth (PCV μ l) of seven soybean genotypes, X2683, x2558, 840-7-3 (840), Maple Arrow (Arrow), Canatto, Nattawa, TNS, in suspension culture (PCV μ l/flask $\pm$ se). Packed cell volume (PCV) was determined by using the formula, $V = 4/3(\pi r^3)$, and counting the number and size (diameter) of embryogenic clumps in a flask (see text) one month after subculture. At least 3 flasks were used for each genotype.

Table 1.5. Effect of maturation on germination of soybean somatic embryos. The somatic embryos developed on MSBM medium were either germinated directly or matured on MBO medium for one month before germination. Embryos were partially desiccated in an empty dish for 5-7 days. Desiccated embryos were then returned to fresh MSBM medium or MBO medium for germination. The values were the average of two experiments.

Medium	Treatment	No. of embryos	No. of embryos germinated	% gemination
MSBM	Desiccated	24	2	8.3
	Control	20	1	5.0
MBO	Desiccated	30	24	80.0
	Control	20	3	15.0

Fig. 1.4. Induction of somatic embryogenesis in soybean.

- a. Cotyledons of 4.5-5.5 mm in size were placed on MSB medium. 1 x.**
- b. A large number of somatic embryos developed from a cotyledon of cv. Maple Arrow. 7 x.**
- c. Some genotypes responded poorly. This picture shows somatic embryogenesis on an immature cotyledon after one month in culture for cv. Nattawa. 5 x.**

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Fig. 1.5. Somatic embryogenesis in a suspension culture of genotype Nattawa.

- a. Establishment of an embryogenic suspension culture.**
- b. After several cycles of selection, an embryogenic suspension culture was established for Nattawa. Figure shows an embryogenic clump. 7 x.**
- c. After transfer to MSBM medium, a large number of somatic embryos were developed. Figure shows embryos developed from embryogenic clumps after one month on MSBM medium. 1 x.**
- d. A closer view of somatic embryos developed from an embryogenic clump. The embryos have a normal morphology. 2 x.**

Fig. 1.6. Somatic embryo germination and conversion.

- a. Embryos developed on MSBM medium were separated from each other and transferred to MBO medium. 2 x.**
- b. After one month on MBO medium, the embryos were partially desiccated in a sealed dish. Desiccated embryos showed vigorous germination upon transfer to fresh MBO medium. 1 x.**
- c. Plants recovered from somatic embryos. 1 x.**
- d. Upon transfer into soil, normal and fertile plants were developed.**

DISCUSSION

Growth regulators are apparently essential for the induction of embryogenesis in soybean. Of several types of growth regulators, 2,4-D and NAA are the most commonly used to induce somatic embryogenesis in soybean (Li *et al.* 1985; Ranch *et al.* 1986; Lazzeri *et al.* 1988; Hartweck *et al.* 1988; Buchheim *et al.* 1989). While NAA-induced embryos are more morphologically normal, 2,4-D induction gives rise to a higher frequency of embryo formation (Lazzeri *et al.*, 1987; Hartweck *et al.*, 1988; Komatsuda, 1992). Establishment of a suspension culture for a genotype is usually preferred due to its practical use, and the embryos with embryogenic tissues induced with 2,4-D were usually used to establish a suspension culture (Finer and Nagasawa 1988; Liu *et al.*, 1992; and Beiley *et al.*, 1993). Liu *et al.* (1992) documented that the embryos and tissues formed on medium containing NAA did not proliferate in suspension culture. In this study, only 2,4-D was investigated for the induction of somatic embryogenesis.

A variety of growth regulators have been evaluated for the efficiency in the induction of embryogenesis in soybean (Ranch *et al.*, 1986). Weaker auxins were found less effective in induction and they suggested that somatic embryogenesis in soybean required stronger or higher concentrations of growth regulators for a response. A moderate to higher level of growth regulators was generally used in induction media for SDPA soybean in various studies (Ranch *et al.* 1986; Parrott *et al.*, 1988, Buchheim *et al.*, 1989; Finer 1989). In this study, a high level of 2,4-D at 180 μM and 248 μM was also found to be the most efficient for somatic embryo induction. The levels of growth

regulators used were generally higher than those normally used in the protocols for many other species. For instance, only 4.5 μM 2,4-D and 0.9 μM kinetin was normally used in the media for inducing somatic embryos in alfalfa.

A minimum of a one-day exposure to 2,4-D induced embryogenesis and increased exposure time also gave rise to an increased embryo number. Continued exposure to 2,4-D beyond 10 days, however, tended to decrease the embryo formation response. This is in accordance with the results reported by Parrott *et al.* (1988) using NAA as the induction growth regulator in SDPA soybean. They found that a minimum of a 3-day exposure to NAA was enough to induce somatic embryogenesis. Reduction of embryogenesis was noticed when cotyledon explants were overexposed to NAA for longer periods. Such a reduction of embryo number associated with extended exposure of explants to auxin was also noticed in other species. Finstad *et al.* (1993) reported a minimum of a 4-day exposure to auxins in alfalfa was required for inducing embryos but exposure longer than 12 days reduced embryo number. Reduction of embryo formation may have resulted from nutrient depletion in media or accumulation of substances which were toxic to embryo induction and development. A shift in sensitivity to auxin exposure in the cells may also be a cause.

There is interactions between genotype and 2,4-D concentration and between genotype and induction time for somatic embryogenesis for genotypes Nattason and X2558, as tested by two-way ANOVA. Therefore, the optimal 2,4-D concentration and induction time for the embryogenesis for a specific genotype may be determined specifically. However, such experiments are very time and labour consuming and

practically difficult. The protocol used for screening genotypes for embryogenesis, therefore, was based on the information obtained in this study as well as from the literature. An induction time of 14 days using 180 μ M 2,4-D concentration was chosen as the standard protocol in this study.

Like many other plants, somatic embryogenesis of soybean adapted to high latitude also showed strong genotype dependence. All twenty genotypes tested formed somatic embryos, indicating that these germplasm types have a high potential for embryogenesis. There was a strong correlation between the percent of responding cotyledons for somatic embryogenesis and the number of somatic embryos per responding cotyledon, which indicated that a genotype showing a higher percentage of responding cotyledons also had a higher capacity to form somatic embryos per responding cotyledon. This was consistent with the observation by Parrott *et al.* (1989) in their study of short-day-photoperiod-adapted soybean. The values obtained in this study using the long-day-photoperiod-adapted germplasm are much higher than those reported by Parrott *et al.* (1989) or Komatsuda (1992) with respect to embryos per responding cotyledon and percentage of cotyledons responding. The highest value reported by Parrott *et al.* (1989) for embryos per responding cotyledon was for cultivar Manchu at 3.14, while this study report 6.85 for X2683-47-1-1. Komatsuda (1992) tested 288 genotypes of *G. max*, *G. soja* Sieb. and Zucc. and *G. gracilis* Skvortz and identified 4 genotypes which produced more than 2.0 embryos per cotyledon with the best responding genotypes, Brownie DL/64/177, Morse, 54.S.30 DL/64/185 and Masshokutoh Kou 502 (*G. gracilis*) showing a response of 4.2, 2.4, 2.0 and 2.2 embryos per cotyledon, respectively. In the present

survey, 85% of the lines tested responded at a higher level than that reported for Manchu or Brownie DL/64/177. Also, with respect to percentage of cotyledons responding, Parrott *et al.* (1989) reported 15% of the genotypes responded above a 50% rate, whereas, in the present study 85% of the genotypes responded above the 50% rate with greater than 70% response being observed in most cases. The reason for this difference is difficult to deduce because the studies are only generally comparable due to protocol differences. However, the differences may be the result of the incorporation of a high 2,4-D level in the induction medium in this study or may reflect a higher regeneration capacity of the more northern-adapted germplasm. Komatsuda (1992) found that the use of 9 μ M 2,4-D rather than 53 μ M NAA in the induction medium resulted in a 33% increase in embryo number. However, not all genotypes responded better with 2,4-D in the induction medium. The latter suggestion that high regeneration rate is linked to northern-adapted germplasm may be a possibility as Harosoy 63 was tested in all three studies and responded only marginally better in the present one (1.84 vs 0.94 for embryos per explanted cotyledon; 48.62 vs 42 for % cotyledons responding) as compared to Parrott *et al.* (1989) but better than in the study by Komatsuda (1992) (0.5 embryos per cotyledon). The low value in the latter study may be due the use of suboptimal levels of sucrose in the induction medium (87 mM rather than 43.5 mM) or a higher medium pH level (7.0 vs 5.8). Ranch (1993) has also noted this trend for a higher somatic embryogenesis response with early maturity groups. A more comprehensive study will be required to test these possibilities.

This study reports that the embryogenesis in LDPA soybean germplasms is

strongly genotype dependent, as reported in SDPA soybean in several studies (Parrott *et al.*, 1989. Komatsuda 1992). However, the question is: Is this an inherent property of a genotype, or are conditions applied in this specific study only favourable for some genotypes? Pedigree analysis with respect to somatic embryogenesis suggests that a genetic basis is present and probably responsible for the embryogenesis response. Table 2.4 shows that with one exception, there are four ancestral lines, 840-7-3, A. K. Harrow, Mandarin, and Altona, in the genetic background of the best responding germplasms. The genotypes which had a very poor regeneration capacity do not have 840-7-3 in their genetic background. Nattason showed high regeneration capacity, however, it has a different genetic background because it does not have any one of four major ancestral lines in its background. A. K. Harrow was identified as a high regeneration genotype (Parrott *et al.* 1989) and the cultivars with high regeneration capacity tested usually have this genotype in their background. However, this genotype also exists in some lines exhibiting poor regeneration. Thus, as reported by Parrott *et al.* (1989), A. K. Harrow background appears to be important but not necessary for high regeneration capacity of LDPA soybean. In this study, 840-7-3, A. K. Harrow and Mandarin were prominent as the ancestral lines in LDPA soybean showing a high degree of somatic embryogenesis. Genotypes A. K. Harrow and Mandarin are introductions from northeast China, whereas, 840-7-3 was originally from Sweden (Delannay *et al.* 1983). Presently, 840-7-3 is an important ancestral line for use in breeding LDPA soybean plants. The pedigree analysis and *in vitro* responses reported here should be useful for studies of *in vitro* regeneration of LDPA soybean.

The above study suggests that a genetic basis is probably involved in the capacity for embryogenesis. Genetic crosses are required to confirm this suggestion. On the other hand, other factors may also be responsible for the genotypic difference. Different genotypes may need different culture conditions for maximum embryo formation. The developmental stage of the cotyledon is also known to be an important factor for induction. Cotyledons of 4.5-5.5 mm of uniform size were used for the embryo induction for all the genotypes. However, the similar size of immature cotyledons does not ensure similar status of cells responsive for induction for a specific genotype. A more comprehensive study is needed to evaluate this.

The number of somatic embryos formed per cotyledon was higher than that reported in many other studies of soybean, implying that the LDPA soybean germplasms probably have a higher capacity for embryogenesis. However, the maximum average number of embryos formed per cotyledon was only about 6. With respect to the hundreds of thousands of cells in surface layer of a cotyledon, only a few cells formed embryos. According to Finer and Nagasawa (1988), a large number of somatic embryos could develop from a suspension culture, if such a culture could be established for a genotype. In this study, seven genotypes from these 20 lines, which represent high, medium, and low responses, were selected to test their response in suspension culture. The responses of a soybean genotype to somatic embryogenesis from immature cotyledon explants in solid induction medium and in suspension culture were found to be different. A genotype which showed a high capacity to form somatic embryos from immature cotyledons on solid induction medium does not necessarily give a better response in

suspension culture for somatic embryogenesis. A high somatic embryogenesis response could be induced in suspension culture for some genotypes which were poor in forming somatic embryos from immature cotyledons. Highly embryogenic, homogenous, and maintainable cultures can only be established for certain genotypes.

Finer and Nagasawa (1988) reported establishment of a highly embryogenic suspension culture in which one soybean genotype, Fayette, was tested. According to their report, careful and strict selection of embryogenic tissue from nonembryogenic tissues was important for the establishment of a highly embryogenic culture. In the present study of comparing 7 genotypes, it was found that selection was needed but appeared not to be a determining factor. Performance of a genotype in suspension culture appeared to be an inherent property for a genotype. Establishment of suspension cultures of Nattawa, TNS, X2558, and Canatto was achieved with ease. Establishment of suspension cultures of Nattawa was repeated four times and they were initiated from separate immature cotyledon cultures over a two-year period. Highly embryogenic suspension cultures were easily established in each attempt and a dissecting microscope was not necessary for selection in the initiation of the culture for this genotype. In contrast, careful and strict selection was unsuccessful in obtaining highly embryogenic cultures for genotype 840-7-4, Maple Arrow, and X2683-47-1-1. Two attempts aimed at establishment of a highly embryogenic suspension culture for Maple Arrow did not succeed. A two-year period of selection to establish a highly embryogenic suspension culture for 840-7-3 was not successful either.

Since only seven of twenty genotypes, which were not randomly selected, were

tested in suspension culture, a statistic test for reverse correlation was not performed. It appeared that there was no obvious reverse correlation for somatic embryogenesis in these two *in vitro* systems. The two best responding genotypes, Nattawa and TNS in suspension culture were from those which showed the poorest response from immature zygotic embryos. Genotypes X2683-47-1-1, 840-7-3, and Maple Arrow which exhibited a highly embryogenic response from immature cotyledons performed poorly in suspension culture. Genotype X2558-13-3-1 showed superior somatic embryogenesis response in both systems.

An embryogenic suspension culture consists of clumps of globular somatic embryos, according to Finer and Nagasawa (1988), Finer (1994). The globular embryos never developed into the cotyledon stage in suspension culture. Thus, the proliferation of the suspension culture is actually through formation of the secondary embryos on the older globular-staged embryos. Accordingly, the genotype dependency of somatic embryogenesis in suspension would appear to be the genotype dependency of producing secondary embryos. Such differential responses of somatic embryo embryogenesis and secondary embryogenesis were noticed previously. In a study of soybean somatic embryo cycling in solidified media, Liu *et al.* (1992) reported that the frequency of explant response, when using zygotic embryo explants and somatic embryos, were reversed in two soybean genotypes. They suggested that there were different processes which were under separate genetic control. Parrott and Bailey (1993) reported that only a limited number of alfalfa genotypes have the ability to undergo recurrent somatic embryogenesis and they proposed that such a ability is a property present in the original

plant, and not one acquired in culture. It is still unknown what genetic factor(s) is responsible for somatic embryogenesis in immature zygotic embryos for soybean. Line 840-7-3 showed high capacity to form somatic embryos from immature zygotic embryos and this line is important and prominent in the genetic background of the genotypes which showed high embryogenesis. However, this line gave rise to a poor response of embryogenesis in suspension culture. It is interesting to note that 840-7-3 is absent in the genetic background of Nattawa and TNS which showed a superior embryogenic response in suspension culture. Thus, different genetic factor(s) may be responsible for the somatic embryogenesis in these two *in vitro* systems.

The embryo yields were different among the four genotypes which showed a higher capacity for somatic embryogenesis in suspension cultures. The embryogenic clumps of Nattawa and TNS were composed of smaller and compact globular embryos. More embryos developed from these types of clumps upon transfer onto embryo development medium. The growth rate of suspension culture of Canatto was the fastest among the genotypes. But the structure of clumps of this genotype was different from the others. The globular embryos in the clumps were larger and the structure was less dense. Thus, the number of globular embryos within a 4-mm clump was lower for this genotype and fewer embryos developed on embryo development medium. The difference of morphology of suspension cultures in genotypes was also noticed by Parrott *et al.* (1994) in a transformation study.

Matured embryos showed a more vigorous germination than those without maturation. This observation is in accordance with that by Buchheim *et al.* (1989),

although the embryos originated from different sources, i.e., cotyledons vs embryogenic suspension culture. Zygotic soybean embryos also require a certain period of maturation for germination (Adams *et al.*, 1983). Buchheim *et al.* (1989) showed that during maturation fresh weight and dry weight of the embryos kept increasing steadily. Accumulation of sucrose and proteins may be associated with the acquisition of vigorous germination. Partial desiccation induced rapid germination and partially synchronized the germination. Without desiccation, germination was sporadic and observed to be a slow process taking place over a period of several months. In addition, desiccation greatly increased the frequency of germination. Partial desiccation of mature embryo-derived calli was also reported to enhance regeneration ability in rice (Rance *et al.*, 1994). Bailey *et al.* (1993b) reported that desiccation with an 85% RH lead to the highest germination frequency over several RH treatments. Desiccation of somatic embryos may also cause a decrease in the endogenous ABA level (Buchheim *et al.*, 1989), (see chapter 3).

CHAPTER 2

CHARACTERIZATION OF A SIMPLE PARTICLE BOMBARDMENT DEVICE FOR GENE TRANSFER INTO ALFALFA AND SOYBEAN CELLS

INTRODUCTION

Gene delivery is one of the key steps for genetically engineering crops using biotechnology approaches. While *Agrobacterium*-mediated transformation is the major method for plant transformation, a number of direct gene transfer techniques have been developed to overcome the limitation of host-range of *Agrobacterium*. Among these, the microprojectile bombardment approach exhibits a number of advantages. For instance, this approach can deliver DNA to thousands of cells in one bombardment. In contrast, microinjection delivers a gene to only one cell each time. Microprojectile bombardment can deliver DNA to any type of cell and tissue, which is in contrast to electroporation and PEG methods which usually use protoplasts as a target (Davey *et al.*, 1989; Nyman and Wallin, 1992; Hoffman *et al.*, 1994; Sagi *et al.*, 1994; Asona and Ugaki, 1994). This approach also offers advantages (Grey & Finer 1993) over *Agrobacterium*-mediated transformation such as the use of more simplified plasmid constructions, elimination of false positives due to *Agrobacterium* persistence in the host tissue and simplified transformation protocols. These properties makes the microprojectile bombardment approach prominent among gene transformation methods.

Since the development of the first particle delivery system (Klein *et al.* 1987), the use of this technique has been spreading and is now being used widely. However, the initial device was less efficient and its operation was complicated. Improvement of this technique has drawn the attention of many laboratories. Since the first report of the device, several different types of bombardment devices have been developed, including

an electrically triggered discharge gun (McCabe *et al.* 1988, McCabe & Christou 1993), pneumatic particle guns (Morikawa *et al.*, 1989; Oard *et al.*, 1990, Seki *et al.*, 1991; Kikkert, 1993; Oard 1993), helium, nitrogen and carbon dioxide powered devices (see Finer *et al.* 1992, Vain *et al.* 1993), and a micro-targeting gun (Sautter *et al.*, 1991, Sautter, 1993). These devices have been developed toward the same goals; that is, more simplicity, safety, accuracy, and a lower cost for DNA delivery (Sanford *et al.*, 1991, 1994; Cray *et al.*, 1994).

Most devices currently in use are commercially available (e.g. PDS-1000/He) and use high pressure helium together with consumable materials, such as rupture disks, macrocarriers, and stopping screens. These devices are expensive and entail a relatively high operating cost due to the consumable materials necessary for each bombardment. In addition, the set up for each bombardment can be complicated and time consuming, especially when many samples need to be bombarded and most material must be presterilized. These properties limit the accessibility to the use of such devices.

A simple particle bombardment device, based on the Particle Inflow Gun (PIG), (Finer *et al.*, 1992, Vain *et al.*, 1993), was built at the Plant Research Centre, Agriculture Canada following directions from Dr. D. C. W. Brown's laboratory. In this system, DNA-coated tungsten particles are accelerated directly by pressurized helium without using consumable materials. This new gun further modified the Particle Inflow Gun by incorporating an electronic system in the device. Thus, the operation can be more precise and efficient. The new device has been found to deliver DNA efficiently into plant cells. This work characterized this new particle bombardment device for gene

delivery in alfalfa and soybean cells and also compared the efficiency of gene delivery into plants cells to that using a commercial gun.

MATERIALS AND METHODS

I. Particle Bombardment Device

The Particle Bombardment Device was designed to directly impel tungsten particles through a partial vacuum into target material using a timed burst of pressurized helium gas. The partial vacuum environment was implemented to reduce aerodynamic drag on the tungsten particles, thereby increasing particle velocities. The system consisted of a thick walled vacuum chamber mounted within a larger enclosure, which also housed associated electronic control circuitry, solenoid and metering valves, plumbing and external connectors. A helium gas supply tank with a pressure regulator was connected to the vacuum chamber via a solenoid valve whose output was connected to a syringe filter unit located within the chamber at the top. A vacuum pump was connected to the chamber via a metering valve and a solenoid valve. Similarly, an atmospheric vent line to the chamber was connected via a metering valve and a solenoid valve. A vacuum gauge was installed on the chamber to monitor vacuum levels. The metering valve on the vacuum line provided control of the flow rate of air being removed from the chamber and thus permitted more precision in setting the selected vacuum level. The metering valve on the vent line permitted a controlled rate of air return into the chamber so that the samples would not be disrupted during venting. In addition, during use, the device was located in a laminar flow bench to allow for aseptic handling of target tissue and provide a source of aseptic air for venting of the vacuum chamber.

The vacuum chamber was constructed of aluminum sheet using welded construction for the sides, back, top and bottom portions of the chamber. A front access door was constructed of Lexan sheet and fixed to the chamber by hinges on one side and latches on the other. An O-ring was embedded in the front aluminum face surface to provide a vacuum seal when the door was latched shut. The interior side walls of the vacuum chamber were machined with horizontal channels to allow fixing of a plate holder shelf, and thus the target material, at various distances from the source of the tungsten carrier particles (syringe filter unit).

Setting the vacuum level in the chamber, firing a burst of helium and venting of the chamber were controlled using switches connected to electronic circuitry which activated the relevant solenoids (For detailed information about the device, see Brown *et al.*, 1994).

A diagram of this device is shown in Fig. 2-1.

Fig. 2.1. Schematic diagram of the Particle Bombardment Device.

- 1: Stainless steel syringe filter unit.**
- 2: Luer-lok needle adapter.**
- 3: Disposable plastic petri dish.**
- 4: Machined plastic plate holder.**
- 5: Vacuum chamber.**
- 6: Vent opening.**
- 7: Swagelog union cross.**
- 8: Omega 70-210 kPa vacuum detector switch.**
- 9: 0-210 kPa vacuum gauge.**
- 10: Asco 2-way solenoid valve.**
- 11: Nupro metering valve.**
- 12: To vacuum pump.**
- 13: Inlet vent.**
- 14: Asco 2-way solenoid valve with 50-999 ms timing circuit.**
- 15: In-line shut-off valve.**
- 16: Matheson dual-stage regulator.**
- 17: Matheson 100-2,800 kPa delivery gauge.**
- 18: Matheson 0-20,700 kPa delivery gauge.**
- 19: High-purity helium gas supply tank.**
- 20: Metal housing.**
- 21: Electronic circuit board.**

II. Preparation of plant materials for bombardment

i) Alfalfa cell culture

a) Initiation of non-embryogenic tissue culture

Callus induction and suspension cultures were initiated as described in Chapter 4. The suspension culture was maintained for an extended time of three weeks, rather than 8 to 10 days as in the standard protocol. After three weeks, instead of transfer to BOi2Y medium, the culture was subcultured with fresh B5mod medium. For subculture, 10 ml of the culture was transferred using a sterile pippet under sterile condition into a flask containing 40 ml fresh B5mod medium. Afterward, the suspension culture was then subcultured every 8-10 days.

b) Preparation of cells for bombardment

The suspension-cultured cells were collected 5 days after subculture by pouring the culture through a sterile 200 μm nylon mesh screen and cells were spread in a thin layer on a 4.25 cm sterile filter paper, pre-moistened with sterile water, in a 60 x 15 mm Petri dish.

ii) Soybean cell culture

The suspension culture of soybean, cv. Nattawa was established as described in Chapter 3 and subcultured every 30 days. The clumps of embryogenic cells were collected fifteen days after subculture and placed on a 4.25 cm filter paper, pre-moistened with sterile water, in a 60 x 15 mm Petri dish for bombardment.

III. DNA preparation

Plasmid PBI121 (Jefferson 1988) (Chapter 4, Fig. 4.1) was used in this set of experiments. DNA was precipitated onto tungsten particles by using the following protocol. A 5 μ l sample of plasmid DNA (1 μ g/ μ l) was added to 25 μ l of tungsten particles (100 mg/ml) and followed by the addition of 25 μ l of 2.5 M CaCl_2 and 10 μ l of 0.1 M spermidine. The suspension was mixed by vortex action for 30 s and left at room temperature for 5 min. After precipitation, 50 μ l of supernatant was discarded and the remaining DNA solution was used for 3 bombardments.

IV. Bombardment

i) Operation of the Particle Bombardment Device

The DNA-coated tungsten particles were loaded in the previously-autoclaved

syringe filter and the target material was placed at the desired location in the chamber, which was swapped with 70% ethanol. The access door was latched shut. Using the VACUUM switch, the vacuum pump was turned on and the vacuum raised to the desired level as indicated by the vacuum gauge (#9 Fig. 2.1). When the desired level was reached (usually 720 mm Hg), the HOLD switch was pressed to de-activate the vacuum pump. The control circuit for the helium solenoid contained a timing circuit which determined the length of time the solenoid was open to release pressurized helium through the syringe filter. The helium solenoid open time was adjustable from 50 to 999 ms. After selecting the appropriate time interval (usually 50 ms), the helium FIRE switch was pressed, causing a burst of helium to accelerate the tungsten particles towards the target material. Following firing, the chamber was vented to atmospheric pressure by pressing the VENT switch which activated the venting solenoid valve. Venting the chamber to atmospheric pressure permitted the access door to be opened and the sample removed.

ii) Bombardment

A 3 μ l sample of the concentrated particle suspension was placed in the centre of the pre-autoclaved screen of a syringe filter (#1, Fig. 2.1). The syringe filter was assembled and screwed into the needle adaptor (#2, Fig. 2.1). Plant tissue in a Petri dish was placed on the adjustable shelf (#4, Fig. 2.1) at a selected distance from the syringe filter. A 1000 μ m metal screen was placed on the top of the Petri dish to reduce

movement of the target tissue. The helium solenoid open time was set to 50-999 ms (usually 50 ms) and the helium pressure regulator (#16, Fig. 2.1) was set to 200-1200 kPa. The samples were bombarded once. Alfalfa suspension-cultured cells were used for optimization of the device and at least 3 replicates were used for each test. Following bombardment, 0.5 ml of the appropriate growth medium was added to the Petri dish and the dish was sealed with Parafilm and incubated at 25° C in dark.

V. Assay for transient GUS gene expression

Approximately 24 hours after the bombardment, 0.5 ml GUS assay solution (Chapter 4) was added to the Petri dish and the cells maintained at 37° C in dark for one day. GUS expression was quantified by counting the number of individual blue spots with a binocular microscope.

RESULTS

I. Non-embryogenic alfalfa suspension culture

An alfalfa non-embryogenic suspension culture was established and used to characterize DNA delivery with the Particle Bombardment Device. The extension of time of the first culture increased cell density and was found to favour the cell division after the first subculture. A lower cell density in the suspension culture resulted in slow cell proliferation and loss of the culture. Thus, a high inoculation density of suspension culture was used. After 3-5 cycles of subcultures the cells lost embryogenic ability. Cells in suspension culture maintained a vigorous cell division and eventually resulted in a developmentally-synchronised and morphologically homogenous cell population. Cell proliferation in such cultures was much faster than that of embryogenic cells in suspension culture and the culture needed to be subcultured every 8-10 days. A longer time for subculture resulted in the death of some cells and subsequently a non-homogenous culture. The non-embryogenic suspension culture could be maintained for a long time by routine subculture.

II. Efficiency of DNA delivery

The Particle Bombardment Device described here was found to deliver DNA efficiently into plant cells. Transient expression of the CUS gene was obtained following

bombardment of both alfalfa and soybean suspension-cultured cells (Fig 2.2). The efficiency of this device for DNA delivery was compared with that of the Du Pont PDS-1000/He gun in a separate set of experiments. No significant difference was found in the efficiency of DNA delivery between these two types of devices (1028 ± 89 [PDS-1000/He] vs. 925 ± 49 blue loci per dish [Particle Bombardment Device], $P=0.31$, T-test) using the optimized settings for each device (Table 2.1). Both devices resulted in variable GUS expression when comparing experiments run on different days (Table 2.2).

III. Factors affect DNA delivery

The efficiency of DNA delivery by this device was studied and optimized by bombardment of alfalfa non-embryogenic cell suspension cultures. Several factors were found to affect DNA delivery, including culture age, bombardment pressure, the distance of sample from the syringe, and the tungsten particle size employed.

Cells at different stages after subculture resulted in different levels of gene expression (Table 2.3). The cells at early stages gave a higher level of gene expression than those at later stages.

Generally, a higher pressure was found to be beneficial for DNA delivery (Fig. 2.3). However, at the high pressures of 1200-1400 kPa the inflow of helium tended to dislodge the samples. When using higher pressures, sample loss was reduced by drying the tissue samples in a laminar flow hood for a short period of time (about 5 min) to reduce the amount of excess medium and by placing a stainless steel 1000 μm mesh

screen on top of the Petri dish. The drying treatment did not appear to reduce the efficiency of DNA delivery (data not shown) but did reduce tissue loss due to dislodgement.

The distance between the DNA loading screen and sample was another factor affecting delivery (Fig. 2.4). The best transfer efficiencies were obtained when samples were placed at a distance of 19 cm using a pressure of 700 kPa. However, if a larger sample size contained in a larger Petri dish is to be bombarded, a longer distance can be used and a higher pressure can be set. For alfalfa suspension culture, the larger particle size of 1.67 μm appeared to give the highest DNA delivery rates (Fig. 2.5).

Table. 2.1. Optimized conditions and GUS gene expression for two different gene guns.

Gun Type	Pressure (kPa)	Distance (cm)	Particle size (μm)	Blue loci/dish*
PDS1000/He	4500	8	1.67	1028 $\pm$ 89
New Particle Gun	1000	19	1.67	925 $\pm$ 49

* A t-test shows that there is no significant difference for the GUS gene expression following bombardment using the optimized conditions by two types of devices ($p = 0.31$).

Fig 2.2. Transient GUS gene expression in alfalfa non-embryogenic suspension culture (a, 5 x) and soybean suspension culture (b, 6 x).

Table 2.2. Comparison of GUS expression, as measured by blue loci per dish, in 5-day-old alfalfa cell suspensions tested at different times using optimized conditions with the Particle Bombardment Device and PDS-1000/He. Bombardment was performed at a pressure of 700 kPa and the samples were placed 19 cm from the loading screen with the Particle Bombardment Device and at 450 kPa and at a distance of 8 cm using the PDS-1000/He. Data represents the mean of 3 replicates $\pm$ the standard error of the mean.

Number of blue spot per dish			
Trial	PDS1000/He	Trial	Particle Bombardment Device
1*	197 $\pm$ 19	5	93 $\pm$ 15
2	236 $\pm$ 40	6	169 $\pm$ 35
3	411 $\pm$ 88	7	234 $\pm$ 40
4	833 $\pm$ 187	8	389 $\pm$ 14
		9	451 $\pm$ 21
		10	642 $\pm$ 26

*Trials listed are independent and arranged in ascending order for convenience only

2

PM-1 3½"x4" PHOTOGRAPHIC MICROCOPY TARGET
NBS 1010a ANSI/ISO #2 EQUIVALENT

PRECISIONSM RESOLUTION TARGETS

Table 2.3. Effect of culture age on transient GUS gene expression in alfalfa cell suspensions. Numbers represent the mean of 3 replicates $\pm$ the standard error of the mean. Data were collected using the PDS-1000/He with 4500 kPa, 1.1 μ m tungsten particles with the target at 8 cm.

Time after subculture (Day)	Number of blue loci per dish
3	246 $\pm$ 76
5	263 $\pm$ 40
7	57 $\pm$ 14
10	24 $\pm$ 4
17	41 $\pm$ 12

Fig. 2.3. Effect of bombardment pressure on GUS expression, as measured by blue loci per dish, in alfalfa cell suspensions. A 5-day-old suspension culture and 1.67 μm tungsten particles were used with the Particle Bombardment Device. The samples were placed at 19 cm from the loading screen. Data are the mean of 3 replicates $\pm$ the standard error of the mean.

Fig. 2.4. Effect of bombardment distance on GUS expression, as measured by blue loci per dish, in 5-day-old alfalfa cell suspensions. A pressure of 700 kPa and 1.67 μm particles were used with the Particle Bombardment Device. Data represents the mean of 3 replicates $\pm$ the standard error of the mean.

Fig. 2.5. Effect of particle size on GUS expression, as measured by blue loci per dish, in 5-day-old alfalfa cell suspensions. Bombardment was at the pressure of 1000 kPa and the samples were placed at 19 cm from the loading screen using the Particle Bombardment Device. Data represents the mean of 3 replicates $\pm$ the standard error of the mean.

DISCUSSION

Alfalfa non-embryogenic suspension culture provided ideal material for characterization of new DNA constructs, new bombardment devices, and transformation efficiency via microprojectile bombardment. First, the culture consists of a homogenous cell population which does not assume a particular development pathway. Thus, the transient expression of the foreign gene will be less affected by cell's morphological change and development pathway, as described in chapter 4. Second, cell division is fast and new material can become available in one week to ten days. Third, the culture provides a large amount of material. One 125 ml flask with 40 ml of culture can usually provide 20-30 bombardments if a 15 x 60 mm Petri dish is used. This characteristic is in contrast to a soybean suspension culture in which a one-month-old culture is usually enough for 1-3 bombardment.

Both PDS-1000/He and the Particle Bombardment Device resulted in variable GUS expression when comparing experiments run on different days. The standard error of the means for a given experiment were in the same range for each device with a strong trend toward lower error values when using the Particle Bombardment Device. A major contributing factor to variable GUS expression can be attributed to the condition of the target tissue. Dramatic differences in GUS expression with time after subculture are apparent with alfalfa cell suspension-cultured cells. Previous work (Atanassov and Brown, 1984) has shown that there are rapid changes (eg. mitotic index, fresh weight, dry weight) during the 14-day period after subculture in alfalfa cell suspensions and that

there is line to line variability that must be accounted for. In particular, the highest mitotic index in alfalfa cell suspensions were shown to occur between day 2 and 6, the same period which showed the highest GUS expression in the present study. However, the data indicate that the reproducibility of response within any given experiment, when the target tissue is uniform, is very good as judged by the standard error of the means. These results emphasize the need for good controls when comparing data generated from different tissue sources or at different times.

The major characteristic of the Particle Bombardment Device is that it accelerates DNA-coated particles directly. When particles hit and penetrate plant cells, the blow from the helium device also affect the materials and cells. Although this impulse did not appear to cause more damage to the cells (Hunold *et al.*, 1994), target tissue could be dislodged, especially when large target tissues, such as soybean suspension-cultured clumps, or alfalfa petiole and leaves, were used. When an alfalfa non-embryogenic suspension culture was placed in a thin layer in the dish, it was much less affected by such an impulse. Withstanding the blow by alfalfa cells could be further increased by sitting the dish open in laminar flow hood for about 5 min to evaporate the excess water. Such treatment was also found to increase GUS gene expression (not shown). A mesh can also be placed above the Petri dish to reduce the impulse and thus keep the tissue from being dislodged or pushed out of the dish (for large materials). The mesh can also adjust the particle travel route and result in a more even distribution in the dish.

The simple Particle Bombardment Device described here has several advantageous properties. First, the only consumable materials needed for bombardment are helium and

tungsten particles and, therefore, the cost is very low for each bombardment. Secondly, since no membrane-rupture system is needed to set up, cleanup and setup time for each bombardment is reduced substantially and operation of the device is very simple. Thirdly, commercially available guns use rupture disks to control bombardment pressure. It was found that the rupture of these disks is often at pressures of 100-350 kPa above or below rated pressure, which results in variation in delivery of particles and, presumably, in GUS expression between replicates. The new particle bombardment device releases exactly at the set pressure; thus, the results obtained among different samples are more consistent (as judged by the standard error of the means) than those obtained by using rupture disks. Better reproducibility will be very useful in the study of transient gene expression. In addition, commercial rupture disks can slide off or may not break at the set pressures, which results in variable expression and lost time. To date, there appears to be no satisfactory solution to this problem with rupture disks. The device described here avoids these problems altogether. In addition to the above properties, this device is inexpensive and simple to build for many laboratories. The cost was approximately 25% of the cost of a yearly lease of a commercial device at the time of construction. The continued development of inexpensive and efficient bombardment devices should facilitate the distribution and utilization of particle acceleration technology for DNA transfer to plant cells.

CHAPTER 3

CHARACTERIZATION OF VARIOUS FACTORS AND CONDITIONS FOR ALFALFA TRANSFORMATION FOLLOWING MICROPROJECTILE BOMBARDMENT

INTRODUCTION

Transformation of alfalfa (*Medicago sativa* L.) by *Agrobacterium* has been reported in several laboratories (e.g. Pezzotti *et al.*, 1991; Schroeder *et al.*, 1991; McKersie *et al.* 1993; Desgagnes *et al.*, 1995, Du *et al.*, 1994), but it appears to be strongly genotype dependent. A biolistic approach, therefore, may be a way to minimize this limiting factor for transformation of alfalfa.

The successful transformation of a species by bombardment is dependent upon many biotic and abiotic factors. Explant choice appears to be one of the most important factors. To date, of reported transformed plant species, success was usually achieved by bombardment of specific explants for each specific plant type. For example, immature embryos appeared to be a superior explant for the bombardment-transformation of several cereal species, including rice, barley, and wheat (Cristou *et al.*, 1991; Li *et al.*, 1993; Ritala *et al.*, 1994; Nehra *et al.*, 1994; Becker *et al.*, 1994). Somatic embryogenesis in alfalfa is highly genotype dependent. However, unlike many other plants in which somatic embryos can only form from one or two specific tissues or organs of the plant (e.g. soybean) in a responsive alfalfa plant a high number of somatic embryos can usually be induced from almost any part of the plant (Brown and Atanassov, 1985; Brown 1988; Bingham *et al.*, 1988, Brown *et al.*, 1989). There has been no systematic evaluation of the range of explant suitability for transformation by microprojectile bombardment.

In this study, different explants were tested; namely, petioles, stems, roots,

leaves, embryogenic callus, and somatic embryos. Different stages in culture were also evaluated to investigate the potential for transformation using these explants as bombardment targets.

Transient GUS gene expression was used in this study. Following bombardment, GUS gene expression could be detected at 48 hrs approximately by a histochemical assay. The substrate solution (X-gluc) was added to the bombarded culture and GUS gene expression was indicated by the blue cells (Jefferson, 1988). The transient gene expression is a fast and easy way to evaluate the explants as potential bombardment targets. This approach has been used to evaluate explants for biolistic-mediated transformation in a number of plants, including cassava (Arias-Garzon and Sayre, 1993), white spruce (Li *et al.*, 1994), and sunflower (Hunold *et al.*, 1995).

MATERIALS AND METHODS

I. Somatic embryogenesis

i) Donor plants

Alfalfa (*Medicago sativa* L. cv. Rangelander, clone A70.34) donor plants were propagated *in vitro* by subculturing individual nodes in 75 ml Schenk and Hildebrandt (1972) medium modified to contain 29.2 mM sucrose, 0.1 μ M indole-3-acetic acid, 0.1 μ M iso-pentyl adenine, 1 % Bactoagar (Difco), pH 5.9, in 10 cm Magenta containers (Magenta Corp., Chicago).

ii) Two-step somatic embryo induction

Petioles were cut 8-10 mm in size and maintained on B5h medium and on SH4K medium (Shetty and McKersie 1993). B5h medium consisted of B5 medium (Gamborg *et al.* 1968), 5.1 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 87.6 mM sucrose, 5.5 mM glutamine, 32.5 μ M glutathione, 95.1 μ M serine, 7.4 μ M adenine, 4.5 μ M 2,4-D, 0.9 μ M kinetin, 0.9 % Bactoagar (Difco) at pH 5.5. SH4K medium consisted of Schenk and Hildebrandt (1972) medium containing 4.5 μ M 2,4-D, 0.9 μ M kinetin, 25 mM proline, 0.4 mM thioproline, 50 mM potassium, 0.25 % Gelrite at pH 5.5. After 14 days on B5h or SH4K media, embryogenic callus was transferred to BOi2Y medium for embryo development. Somatic

embryos were recorded 20-days after transfer to BOi2Y medium. BOi2Y medium consisted of Blaydes medium (Blaydes, 1966; Bingham *et al.* 1975) modified to contain 3% sucrose, 0.2% yeast extract, 100 mg/L myc-inositol, and 1% agar.

iii) Three-step somatic induction

Twenty days after introducing the petioles into culture in B5h medium, somatic embryos with developed callus from petioles were transferred into liquid B5 medium modified to contain 4.5 μM 2,4-D, 0.5 μM naphthalene acetic acid, and 58.4 mM sucrose (B5mod). One and one half to two grams of tissue was usually cultured in a 125 ml flask containing 40 ml of medium. The suspension culture was shaken on an orbital shaker at 130 RPM at 25°C under a 16-hr photoperiod of fluorescent Sylvania "Cool White" light at 50 $\mu\text{mol m}^{-2}\text{s}^{-1}$. After 6-8 days in suspension culture, the cell suspension was poured through a sterilized 1000 μm mesh screen and a 200 μm mesh screen consecutively. The culture retained on the 200 μm screen contained small embryogenic cell clusters and was evenly spread on a sterile piece of 200 μm nylon screen which was previously placed on growth regulator-free BOi2Y solid medium in 30 x 15 mm Petri dishes.

iv) Secondary somatic embryo induction

After about fifteen days on BOi2Y medium, cotyledonary-staged embryos were

transferred to MBO medium, as described in Chapter 1, for secondary somatic embryo formation.

A diagram showing the embryogenesis of alfalfa is illustrated in Fig. 3-1.

II. Non-embryogenic suspension culture

See Chapter 2.

III. Preparation of plant materials for bombardment

Non-embryogenic suspension culture at different times after subculture was collected by pouring the culture in a flask onto a 200 μm nylon mesh supported by a funnel in a flask. The collected cells were spread in a thin layer on a filter paper which was then transferred to a 60 x 15 mm Petri dish and 100 μl of B5mod medium was added into the dish.

Petioles, stem, and roots, cut 8-10 mm in size, and intact leaves were placed and maintained on B5h medium prior to bombardment. Five to 7 petioles, stems, roots, or six leaves were cultured in each Petri dish (60 x 20 mm) containing 20 ml medium. The dish was sealed with Parafilm. Culture conditions were maintained at 25°C under a 16-hr photoperiod with a light intensity of 25 $\mu\text{mol m}^{-2}\text{s}^{-1}$; Sylvania "Cool White" fluorescent tubes.

Fig. 3-1. Diagram showing the tissue culture protocol in alfalfa.

- I A two-step embryo formation protocol.**
 - II A three-step embryo formation protocol.**
-
- 1 Callus induction.**
 - 2 Cell proliferation in suspension culture.**
 - 3 Embryo development.**
 - 4 Embryo germination.**
 - 5 Formation of secondary somatic embryos.**
 - 6 Plant recovery.**

Eight to ten petioles, stems, roots, calluses, or six leaves at different times after being placed on B5h medium were placed on a sterilized filter paper premoisturized with 200 μ l liquid B5h medium in a 30 X 15 mm Petri dish.

Proembryogenic tissue and somatic embryos separated from suspension cultures were transferred with supporting mesh to an empty dish. One hundred microlitres of liquid BOi2Y medium was added into the dish.

IV. Bombardment

The plasmid PBI221.23, as described in chapter 4, was used in the study. The DNA coating procedure was the same as described in chapter 4. The culture was bombarded with tungsten particles using the PDS-1000/He apparatus. The gap distance (distance from rupture disk to macroprojectile) was 15 mm; the particle flight distance was 7.5 mm; target distance (the distance from stop screen to plant tissue) was 45 mm. A piece of 500 μ m mesh was placed above the Petri dish for a better particle distribution. The tissue was bombarded once. At least 3 replicates (dishes) were used for each condition. The bombarded culture was then returned to the appropriate medium for 24 hrs. GUS expression was tested by histochemical assay (Jefferson 1988). The GUS assay was as described in chapter 4.

RESULTS

I. Somatic embryogenesis

i) Two-step somatic embryo induction

Embryogenic callus formed on petioles after 14 days on B5h or SH4K medium. These embryogenic calluses were then transferred to BOi2Y medium for embryo development. After 10 to 14 days on BOi2Y medium, somatic embryos formed (Fig 3.2). An average of 6 somatic embryos from each callus was induced on B5h medium and 40-50 embryos per callus were induced on SH4K medium.

ii) Three-step somatic embryo induction

Embryogenic callus was induced on B5h medium for 20 days. The embryogenic cells proliferated in suspension culture for 6-8 days before transfer to BOi2Y medium for embryo development. After 10 to 15 days on BOi2Y medium, hundreds, in some cases over one thousand, embryos could form from the tissue in a single dish (Fig. 3.3). It was estimated that 50 to 100 somatic embryos could be produced from an original petiole explant.

iii) Secondary somatic embryo induction

After about 15 days on BOi2Y medium, embryos had developed to torpedo and cotyledonary stages. Upon transfer to MBO medium, embryos either germinated, or started to form secondary embryos, depending on individual embryos. Once an embryo was identified to be able to produce secondary embryos, such ability could be maintained for a long time by transferring cotyledonary-staged secondary embryos to fresh MBO medium. In the early cycles of secondary embryo formation, the number of secondary embryos formed on an old embryo varied greatly. After several cycles (i.e. 3-5), the secondary embryo formation became stable and about 30-40 secondary embryos could be formed from a single old embryo.

II. Bombardment conditions

First, some conditions for DNA delivery were tested by bombardment of petioles at day 5 in B5h medium. Preliminary experiments showed that bombardment of petioles at day 5 gave the highest GUS expression. Two target distances (4.5 cm and 11 cm) were tested and the results showed the GUS expression was better at a distance of 4.5 cm from the stop screen (Table 3.1). A particle size of 1.3 μm gave the best GUS gene expression among the 3 sizes of particles (1.1, 1.3, 1.6 μm) tested (Fig. 3.4). PSI 650 and 1100 gave a similar level of the GUS gene expression (Table 3.1). In later experiments, PSI 650 was used as bombardment pressure to reduce the possible damage to the cells.

Fig. 3.2. Somatic embryogenesis of alfalfa with a two-step protocol.

a: Embryogenic callus developed after 14 days in culture. Notice the difference of the calluses induced in two media. 1 x.

Left: B5h.

Right: SH4K.

b: After transfer of the calluses to BOi2Y medium different numbers of somatic embryos were formed from calluses induced in two different media. 1 x.

Left: B5h.

Right: SH4K.

Fig. 3.3. Somatic embryogenesis of alfalfa with a three-step protocol.

- a. Petioles placed in culture on B5h medium. 1 x.**
- b. Callus developed from petioles after 2-3 weeks in culture. 1 x.**
- c. Sieved culture was placed on BOi2Y medium, after suspension culture. 1.5 x.**
- d. Development of a large number of somatic embryos after two weeks on BOi2Y medium. 1.5 x.**

Table 3.1. GUS gene expression in alfalfa petioles at two bombardment distances and two pressures using the PDS-1000/He particle bombardment device.

		GUS gene expression (blue spots/petiole)
Pressure* (psi)	650	6.8 ± 1.6***
	1100	6.1 ± 1.3
Distance** (cm)	4.5	7.9 ± 1.6
	11.0	3.1 ± 0.6

* Bombarded at the distance of 4.5 cm from the stop screen.

** Bombarded at the pressure of 650 psi.

*** Mean ± standard error of the mean of at least 30 petioles for each condition.

GUS expression in the petioles bombarded at 45 mm and 100 mm from stop screen is significantly different ($p=0.0048$, t-test).

Fig. 3.4. Effect of particle size on GUS expression, as measured by blue spots per petiole, in 5-day-old alfalfa petiole in culture. Vertical lines indicate the standard error of the mean of at least 30 petioles for each condition.

III. GUS gene expression and cell division activity

Unlike the suspension culture in soybean in which the culture is maintained by monthly subculture and the cells retain their embryogenic ability for several years, alfalfa suspension culture lost its embryogenic ability after three to five rounds of subculture. Such culture was used to investigate GUS gene expression and cell division activity following bombardment.

Differences in GUS expression with time after subculture were apparent with alfalfa non-embryogenic suspension-cultured cells. Bombardment of the culture at an early stage after subculture, ie. at day 3 and day 5, resulted in the highest GUS gene expression. Bombardment of the cells at later times after subculture gave rise to a lower GUS expression (Table 2.3, Chapter 2).

IV. Explant effect

Petioles, stems and roots after 5 days in B5h medium were subjected to bombardment. GUS expression was 7.6, 6.5, and 2.2 blue spots per petiole, stem, and root respectively. GUS expression in these three types of explants was not significantly different ($p = 0.27$) as measured by One-Way-ANOVA. Leaves of two genotypes, A70.34 and 11.9 were also subjected to bombardment. The GUS expression in the leaves of A70.34 was lower than in that of 11.9 (Table 3.2). Leaves of A70.34 were also bombarded using 900 psi, 1100 psi and 1300 psi. The alternative bombardment

conditions still resulted in low GUS expression in A70.34 (not shown). Leaves were then pretreated with ethanol before bombardment to simulate the tissue disinfectant procedure. After treatment, the GUS expression pattern was reversed; that is, the GUS expression in A70.34 was increased dramatically but decreased in the leaves of 11.9 (Table 3.2).

V. Culture media effect

In the two-step somatic embryo induction, the B5h medium usually produced 5-8 somatic embryos per petiole. Somatic embryo production was dramatically increased if petioles were cultured in SH4K medium (40-50 per petiole) (Fig 3.2). Surprisingly, the GUS expression was consistently and extremely low in the petioles cultured with SH4K medium (Fig. 3.5).

VI. Developmental effect

Petioles at different times during culture were subjected to bombardment. GUS gene expression was low at the early stages of culture but increased with the time in the culture. Bombardment of petioles at day 5 gave the highest GUS gene expression (Fig 3.5.). After about 15 days in culture, petiole cells had dedifferentiated and callus had formed from the petioles. Bombardment of embryogenic calluses developed from petioles, however, resulted in a decreased GUS gene expression. Callus at day 17 on

B5h was also crushed and placed in a thin layer on a sterile filter paper prior to bombardment. The callus was bombarded at the distance of 4.5 and 12 cm from stop screen using 650 and 1100 PSI pressure respectively. GUS expression was still low with these bombardment conditions.

After suspension culture, the cultured cell population consisted of embryogenic cells and tiny globule somatic embryos which were about 0.1 to 0.5 mm in size. The culture was then transferred onto BOi2Y medium for embryo development. The embryogenic tissues and the somatic embryos on BOi2Y medium at different stages were also subjected to bombardment. It was found that the GUS gene expression in the embryogenic unorganized tissue was the lowest. After 3 days, some GUS expression was detected, but mainly on the small green developing somatic embryos. After about 10 days, when somatic embryos had developed into torpedo and cotyledon stages, the GUS expression increased dramatically (Table 3.3).

Table 3.2. GUS expression in leaves of two genotypes: A70.34 and 11.9 with and without EtOH treatments. The GUS gene expression was measured two days after bombardment.

Genotype	GUS gene expression (blue spots/petiole)	
	Treatment	
	-EtOH	+EtOH
A70.34	3.7 $\pm$ 1.5	40.8 $\pm$ 11.0*
11.9	43.6 $\pm$ 12.9	4.2 $\pm$ 1.1

* Mean number of blue spots per leaf $\pm$ standard error of the mean of at least 3 leaves.

Fig. 3.5. GUS gene expression in petioles and calluses cultured in B5h and SH4K media and bombarded at different times in culture. The vertical lines (+ & o) indicate the standard error of the mean for at least 30 petioles for each condition.

(+). GUS expression on petioles cultured on B5h medium.

(o). GUS expression on petioles cultured on SH4K medium.

(Δ). Growth of petioles on B5h medium over 20 day induction. The vertical lines indicate the standard error of the mean of at least 5 petioles for each condition (These petioles were not bombarded). The growth on SH4K was not measured but appeared the same as on B5h.

Table 3.3. GUS gene expression on proembryogenic tissue and somatic embryos on BOi2Y medium at different stages.

Bombardment target	Time on BOi2Y (days)	No. of blue spots/dish[*]
Proembryogenic tissue	0	0.7
Proembryogenic tissue development somatic embryos	3	7.3
Somatic embryos	12	810^{**}

^{*} Mean number of blue spots of at least 3 replicates (plates).

^{**} Bombarded somatic embryos were randomly selected and blue spots on each embryo were counted. The average number of blue spots per embryos was then multiplied by 100, which was the estimated number of somatic embryos per dish.

VII. Genotype effect

The experiment was also carried out to test if microprojectile bombardment can be used as a general approach for the transformation of embryogenic lines of alfalfa with different genetic background. Petiole is one of commonly used explants for embryogenesis in alfalfa and was used in the experiment. Petioles of ten different alfalfa genotypes, namely, A70.34, 11.9, AK.1, Europe, F1.1, X10, X13A, X6A, WTRF2-21 (W-21) and WYRF2-26 (W-26), were subjected to bombardment. These ten genotypes were selected from a wide range of genetic backgrounds and all were highly embryogenic as tested in several laboratories using different types explants. Line A70.34 is a commonly used line for somatic embryogenesis in a number of laboratories, but this genotype appeared to be difficult to transform with *Agrobacterium* (Du, *et al.*, 1994). Line 11-9 is the best responsive genotype screened for *Agrobacterium* transformation (Desgagnes *et al.*, 1995). WTRF2-21 and WYRF2-26 are diploid plants which showed high embryogenesis using two-step protocol and the remaining are tetraploid plants. Bombardment of these ten genotypes appeared to give a similar level of GUS gene expression (Fig. 3.6). One-way variance analysis indicates that the gene expression in these ten genotypes is not significantly different ($F = 0.843$, $P > 0.05$, Table 3.4). The average number of GUS gene expression in the petioles of these ten genotypes is as follows: A70.34: 6.25 ± 0.77 ; 11.9: 3.95 ± 1.21 ; AK.1: 4.57 ± 0.97 ; Europe: 6.39 ± 1.13 ; F1.1: 7.33 ± 1.40 ; X10: 6.57 ± 0.99 ; X13A: 5.93 ± 1.01 ; X6A: 6.11 ± 0.91 ; WTRF2-21: 6.94 ± 0.93 and WYRF2-26: 5.80 ± 0.99 .

Table 3.4. One-Way ANOVA analysis of GUS gene expression in petioles of ten alfalfa genotypes following bombardment. The results indicate that there is no significant difference for GUS gene expression among the genotypes. At least 30 petioles were used for each genotypes.

	Mean Square	F-Value*
Between	24.70	0.843
Within	29.30	

* The exact p value was not able to be identified because the value is out of the range of the F-table.

VIII. Transient vs. stable expression

The fate of the cells which transiently expressed the foreign gene was determined by bombardment of petioles. About 200 petioles were bombarded, with ten petioles each per bombardment, at day 5 after culture. A sample of at least 17 petioles was randomly selected at different times ranging from 0 hr to 15 days following bombardment and a GUS assay was then performed. The GUS gene expression was low immediately after bombardment, as shown at 0 hr (Fig 3.7). A short time of incubation post-bombardment increased GUS expression, as indicated at 8 hrs. After 24 hrs, the GUS gene expression reached the highest level. Such high level GUS expression was only detected 24-48 hrs following bombardment. Thereafter, the GUS expression decreased quickly. By 15 days after bombardment, only a few cells still expressed the GUS gene.

GUS gene expression in different explants of alfalfa following bombardment is shown in Fig. 3.8.

Fig. 3.6. GUS gene expression in petioles of 10 different genotypes. Vertical lines indicate the standard error of the mean of at least 30 petioles for each genotypes.

Fig. 3.7. GUS gene expression in petioles at different times after bombardment.
Vertical lines indicate the standard error of the mean of at least 17 petioles.

Fig. 3.8. GUS gene expression in different explants of alfalfa following bombardment.

- a. In petioles. 6 x.**
- b. In leaves of two genotypes: A70.34 and 11.9. The leaves were treated in ethanol briefly before bombardment. A70.34 (small leaf) showed higher GUS expression than that observed in 11.9 (large leaf). 5 x.**
- c. In developed somatic embryos. 6 x.**
- d. In non-embryogenic suspension culture. 3 x.**

DISCUSSION

Microprojectile bombardment provides a novel approach for the transformation of many plant species. Nevertheless, transformation frequency via this approach remains low. Very often, bombardment of hundreds of explants results in only one or a few transgenic plants (Becker *et al.* 1994; Nehra *et al.*, 1994; Ritala *et al.*, 1994; Brar *et al.*, 1994). When an organ, a piece of tissue, or a group of cells are subjected to bombardment, only a small number of the total cell population can be hit by particles. Of these cells, only part express the introduced genes. About 1% or less of the cells that transiently expressed the foreign gene can transit into stable expression (Finer and McMullen 1991; Hunold *et al.*, 1994). Of these stable transformed cells, only a small number of the cells are capable of forming plants, depending on the *in vitro* system of the species. Because of these unfavourable rates, some abiotic and biotic factors affecting gene transfer and integration need to be studied and protocol conditions must be optimized before an efficient transformation system can be established.

Selection of suitable explants for bombardment is one of the most important factors for stable transformation. To date, of transformed plant species, success was usually achieved by bombardment of specific explants for specific plant types. For instance, immature embryos appeared to be a superior explant for the biolistic-mediated transformation of several cereal species, including rice, barley, and wheat (Li *et al.*, 1993; Ritala *et al.*, 1994; Nehra *et al.*, 1994; Becker *et al.*, 1994). This study investigated responses of different alfalfa explants to bombardment. The GUS gene was

used as a reporter gene under the control of a CaMV35S promoter. The CaMV35S promoter is generally regarded as an ubiquitous promoter and has been reported to express in various plant organs and tissues.

The petiole, stem, and root can all be used as explants for inducing somatic embryos, although petioles are the routinely used explant. These explants are morphologically similar and were grouped together for the evaluation. Bombardment of these explants resulted in the similar level of GUS expression. This indicated these explants had similar physiological characteristics for gene expression following bombardment. These explants, however, are very thin and the surface area for the bombardment is very small. In addition, preparation of a large number of these explants for bombardment is time and labour consuming. Leaf explants, on the other hand, minimize these limitations. GUS expression patterns in the leaves of two genotypes were different when subjected to different pre-treatments, e.g. with or without immersion in ethanol alcohol. This indicates that some physical factors, other than physiological or biochemical factors may affect transient gene expression. Ethanol treatment may have removed or destroyed some surface structure which may prevent efficient gene delivery or expression. The different expression in two genotypes described here is different from that reported by Moore *et al.* (1994) in which transient expression of a reporter gene in zygotic cotyledons of soybean was genotype specific following bombardment. They suggested that some biochemical characteristic, other than physical factors, were responsible for the different expression. A70.34 has been reported to be recalcitrant for transformation by *Agrobacterium* (Du *et al.*, 1994). Genotype 11.9 is able to be

transformed by *Agrobacterium* (Desgagnes *et al.*, 1995) and leaves were used as explants for *Agrobacterium* infection. Wounding in explants is one of the critical factors for infection and successful transformation via *Agrobacterium* (Weising *et al.*, 1988; Hooykaas, 1989). The different expression in leaves of two genotypes resulting from different treatments suggests that the difference in surface structure in different genotypes may affect *Agrobacterium* infection. This may partially account for genotype specific transformation by *Agrobacterium*.

Only a few cells within an explant are undergo dedifferentiation and formation of new plants via either somatic embryogenesis or organogenesis, when induced *in vitro* with auxins and cytokinins. Delivery of the gene into these specific cells is the first step towards achieving transformed plants. Currently, bombardment is usually a random event in hope of hitting the cells within the explant capable of forming new plants. Five to seven somatic embryos usually formed per petiole explant induced in B5h medium for genotype A70.34 via the two-step embryo induction. Thus, the chance of hitting these embryogenic cells is extremely low. Shetty and Mckersie (1993) reported that addition of proline, thioproline and a higher than normal level of potassium in induction basic medium greatly increased the number of embryos in alfalfa petiole culture. It was speculated that bombardment of petioles on SH4K medium would increase the chance of hitting embryogenic cells. Surprisingly, the GUS expression was consistently and extremely low in the petioles cultured in this medium and bombarded at different times during the induction period of fourteen days. Petiole cells during this period of time exhibited an active division, when cultured in B5h medium. However, petiole cells

cultured in these two media may have assumed different metabolic pathways. The calli induced in B5h medium were yellow-brown in colour but calli formed on SH4K were cream in colour. Change in the physiological and biochemical status of the cells may have resulted in different receptivities to bombardment. In other words, the induction and development of a cell's competence for somatic embryogenesis does not necessarily induce the cell's competence for transient gene expression via bombardment. Petioles are routinely used explants for inducing somatic embryos in the *Medicago* species due to their consistency in embryo production. Bombardment of petioles over a 20-day period in B5h induction medium (the first step of a three-step embryo formation protocol) resulted in different levels of GUS expression. It was clear that there was a stage at which cells were most responsive for bombardment. Cells appeared to need some induction or stimulation to develop their response to bombardment, since lower GUS expression was observed when the petiole explants were bombarded immediately after being introduced into culture. A three- to five-day induction period was necessary for alfalfa petiole cells to transiently express foreign genes. In a study of the cell's competence for somatic embryogenesis in alfalfa, Finstal *et al.* (1993) showed that a minimum of a four-day exposure to 2,4-D was required for cells to develop competence for somatic embryogenesis. Although the timings for the development of a cell's competencies for somatic embryogenesis and high level of gene expression overlapped at the similar stage, a cell's competence for embryogenesis appeared to be different from that for gene expression. Preculture of explants leading to higher expression was also reported previously in barley (Kantha *et al.*, 1989), *Arabidopsis thaliana* (Seki *et al.*,

1991) and sunflower (Hunold *et al.*, 1995). Moore *et al.* (1994) found that GUS expression was only limited to epidermal and subepidermal cell layers of soybean cotyledons regardless of bombardment pressures applied. In our study, GUS expression was presumed to be in these two cell layers. Wenzel and Brown (1991) reported that subepidermal and epidermal layers of alfalfa petioles, from which somatic embryos formed, started to divide after 72 hrs in culture. This time period corresponds to the highest level of GUS expression, which implies that incorporation and (or) expression of foreign gene is related to cell division activity.

After about 10 days in culture, petiole cells had dedifferentiated to form callus, primarily from the three epidermal and subepidermal cell layers (Wenzel and Brown 1991). Bombardment of embryogenic calluses developed from petioles, however, resulted in low GUS gene expression. It was suspected that the structure of callus was looser than that of a petiole, which affected efficiency of gene delivery. However, GUS expression was not increased in crushed callus with modified bombardment conditions. The fresh weight of petiole/callus showed a steady increase between day 10 and day 20 in culture (Fig 4-5), which presumes an active cell division. The surface area of individual explants was increased dramatically (about 5-10 times) which gave a greater bombardment target area. Thus, the low expression of the GUS gene in calluses following bombardment reflects a low response of the callus cells to bombardment. Such low expression in embryogenic calluses in alfalfa was also noticed by F. Pereira (personal commun., Univ. of Guelph). Thus, it can be speculated that an active cell division favours foreign gene expression, as indicated in non-embryogenic homogenous

culture following bombardment, in which cells simply proliferate without assuming a particular developmental pathway (see Chapter 5). Transient gene expression is more affected by cell's developmental pathway. If a cell's metabolism pathway is not favourable, an active cell division alone is unable to stimulate foreign gene expression.

Bombardment of somatic embryos is another way to achieve stable transformation and several laboratories have made such efforts in Norway spruce and white spruce (Robertson *et al.*, 1992; Bommineni *et al.*, 1993). Since the shoot meristem of somatic embryos is exposed, delivery of genes into meristem cells and development of these cells would be more likely to result in stable transformation. Stable transformation via bombardment of meristem cells of zygotic embryos and shoot apices has been reported in soybean (McCabe *et al.* 1988). Alternately, development of secondary embryos from primary bombarded embryos also provides a way to achieve stable transformation. Differential GUS expression was also observed in the cultures bombarded at the different stages on BOi2Y medium. Embryogenic tissue and tiny globular embryos showed a lower response to bombardment. The response was dramatically increased when the embryos started to mature. Lower expression of the GUS gene in proembryogenic tissue than in mature embryos was also noticed previously in Norway spruce (Robertson *et al.*, 1992). The property, status, and nature of cells, thus, could have a dramatic effect on gene expression. The reason for lower transient gene expression in some tissues is not known. Arias-Garzon and Sayre (1993) reported a low transient gene expression in cassava roots following bombardment. Since high levels of DNase activity was found in the roots, they postulated that the high levels of DNase activity compromises the

integrity of the transforming DNA resulting in reduced transformation efficiency. The high GUS gene expression in alfalfa was obtained in somatic embryos which were under development from the torpedo to the cotyledonary stage. Such a time period will favour the idea of transformation via secondary embryo formation, since secondary embryos could be induced from these late-staged embryos if they are transferred to MBO medium.

The genetic background of cultivated alfalfa is complicated and includes several subspecies and land races with diploid and tetraploid plants (Brown and Atanassov, 1985; Meijer and Brown 1985; Brown, 1988; Brown et al., 1989). Bombardment of ten genotypes of alfalfa with various genetic backgrounds gave a similar level of GUS gene expression. This indicated that most alfalfa genotypes, if not all, could have a similar receptivity to bombardment, when petioles are used as explants. Nevertheless, different responses of genotypes to bombardment have been observed. Moore *et al.* (1994) reported that transient expression of a reporter gene in zygotic cotyledons of soybean was genotype specific with various bombardment conditions. Duchesne and Charest (1991) bombarded 10 *Picea mariana* embryogenic lines from six different open-pollinated families and showed comparable transient expression of GUS gene in all but one line. These findings suggest that even though bombardment is genotype independent, as proposed in the early stage of gene gun development, different genotypes may be affected by different developmental and physiological stages, which in turn affect bombardment receptivity and (or) need different treatments or bombardment conditions with respect to certain explants.

The rapid decline of GUS gene expression following bombardment indicates that

the majority of the cells did not survive bombardment or transient expression was unable to transit to stable expression. The quick disappearance of GUS gene expression appears to be a common phenomenon following bombardment (Duchesne and Charest, 1992; Bommineni *et al.* 1994). Bommineni *et al.* (1994) reported that by day 14 after bombardment transient GUS expression dropped to 3 blue foci from 202 foci per plate in stage I embryo culture of black spruce. The reason for the rapid decrease of GUS expression is still not well understood. The damage to cells incurred following bombardment probably is an important reason. Hunold *et al.* (1994) reported that only about 1-2% of tobacco leaf cells containing particles survived the bombardment. In this study, some of the cells still expressed the GUS gene in the later stages of the culture (i.e. 15 days after bombardment), which indicated the possibility of stable transformation. Increase of survival rate of bombarded cells is probably one of the most important tasks for increase of transformation frequency in this system. Less damage to cells by using the Particle Inflow Gun (Finer *et al.*, 1992; Brown *et al.*, 1994) has been suggested (Hunold *et al.*, 1994). Improvement of culture conditions may also improve the survival of bombarded cells (Russell *et al.*, 1992; Bommineni *et al.*, 1994).

CHAPTER 4

TRANSFORMATION OF LONG-DAY-PHOTOPERIOD -ADAPTED SOYBEAN VIA MICROPROJECTILE BOMBARDMENT

INTRODUCTION

Although soybean transformation has been reported in several laboratories using various techniques, none of them has been demonstrated to be consistent and efficient.

Combination of somatic embryogenesis with a microprojectile bombardment approach provides another way for soybean transformation and overcomes some drawbacks present in other approaches used, such as the host range limitation of *Agrobacterium* and chimeric plants from bombarded meristems. Finer and McMullen (1991) reported transformation of a soybean cv. Fayette by bombardment of an embryogenic suspension culture. The plants recovered were truly transformed and selection for non-chimeric plants in the next generation was not needed.

In spite of the advantages of this method, general use of this approach has been progressing slowly. Subsequent efforts using the same system for stable transformation was only demonstrated successfully in the same variety, Fayette (Finer *et al.*, 1992; Sato *et al.*, 1993). Recently, Parrott *et al.* (1994) demonstrated extension of the technique to another variety. They bombarded suspension cultures of three soybean genotypes but only one genotype F376 generated a single transgenic line. The transformation efficiency in their system was extremely low and a large amount of culture with over 20 bombardments resulted in only one transformation event (Dr. Parrott, person. commun.). Although the T₁ progeny was obtained, the expression of the transgene and the phenotype of the plants was not stable.

Currently, soybean genetic engineering remains inefficient and definitely not

routine, especially when compared to some other crops. The number of genes which plant breeders and other scientists need to engineer into soybean vastly exceeds the available engineering capacity. Reliable and efficient techniques, thus, still need to be developed.

To date, all the studies on soybean transformation, even with limited success, have been with the genotypes and germplasms of short-day-photoperiod-adapted soybean. It is known that the *in vitro* system and transformation approach varies vastly among germplasms, even genotypes. Studies were performed to investigate the potential capacity of long-day-photoperiod-adapted soybean to be transformed by microprojectile bombardment. The embryogenic suspension culture system developed in Chapter 1 was used in this transformation study. Two plasmids carrying different selection marker genes, which have been successfully used in different soybean transformation systems, were used to compare their selection efficiency.

MATERIALS AND METHODS

I. Preparation of tissue cultures for bombardment

Embryogenic suspension cultures of soybean cv. Nattawa, TNS, X2558-13-3-1, and Canatto were established as described in the previous chapter. The cultures were maintained by monthly subculture in Ms medium (Chapter 1). For subculture, two clumps of embryogenic tissue, 4 mm in size were transferred to 40 ml Ms medium.

II. Plasmids

Two plasmids, PBI121 (Jefferson *et al.*, 1986, Jefferson, 1988) and PBI221.23 (Lonsdale *et al.*, 1990), were used in the studies (Fig. 4.1). PBI121 carries the GUS gene and NPTII gene under control of a CaMV35S promoter and a NOS promoter respectively. NPTII confers resistance to the antibiotic kanamycin. PBI221.23, (8 kb) (from Dr. M. W. Bevan, John Innes Institute, Colney, Norwich, England), carries the GUS gene and HYG gene and both genes are under control of CaMV35S promoters separately. The HYG gene confers resistance to the antibiotic hygromycin (B). In the early experiments, plasmid PBI121 was used and in later experiments plasmid PBI221.23 was used.

Fig. 4.1. **The maps of plasmids PBI121 and PBI221.23. Diagrams show the regions of interest.**

PBI121

PBI221.23

III. Plasmid DNA isolation

Escherichia coli strain DH5- α (BRL) was transformed with plasmids using a standard CaCl_2 method provided by the company. The bacterium was grown overnight in LB broth supplemented with 100 mg/L kanamycin or ampicillin. The plasmids were isolated by the standard alkaline lysis procedure and purified by the CsCl method (Sambrook *et al.* 1989).

IV. Tungsten particle preparation

Sixty mg of tungsten particles ($1.67\ \mu\text{m}$) were placed in a microcentrifuge tube and vortexed in 1 ml 100% ethanol alcohol for 1 min. The particles were centrifuged for 1 min and ethanol was removed. The particles were washed with sterile distilled water twice and resuspended in 50% sterile glycerol solution. A $50\ \mu\text{l}$ aliquot was distributed into a microcentrifuge tube.

V. DNA preparation

DNA coating was according to Klein *et al.* (1988). To a $50\ \mu\text{l}$ tungsten particles aliquot in a 1.5 ml microfuge tube, $5\ \mu\text{l}$ DNA ($1\ \mu\text{g}/\mu\text{l}$), $50\ \mu\text{l}$ CaCl_2 (2.5 M) and $15\ \mu\text{l}$ spermidine (0.1 M) were added while vortexing. The mixture was vortexed for 2 min at room temperature and then the tube was set on the bench for 5 min. The supernatant

was removed. The DNA-coated particles were washed with 100% ethanol twice and resuspended in 40 μ l 100% ethanol. Eight microlitres of the suspension was pipetted onto a macrocarrier for bombardment.

VI. Bombardment

The PDS-1000/He apparatus was used in the experiments (Fig. 4.2). The apparatus was sterilized with 70% EtOH. Stop screens and macrocarriers were sterilized by autoclaving. Rupture disks and macrocarriers were sterilized by soaking in 70% EtOH for 30 seconds and air dried in a sterile laminar flow hood.

The bombardment pressure was 650 psi and the vacuum level was 28 in. Hg (710 mm Hg). The gap distance (distance from rupture disk to macroprojectile) was 0.21 cm; the particle flight distance was 0.75 cm; target distance (the distance from stop screen to plant tissue) was 11.5 cm.

The cultures from one flask, about 15 days after subculture, were collected and gently crushed into smaller clumps. The clumps were divided into three parts and each was placed on a sterile filter paper in a 15 x 60 mm Petri dish. Excess medium was removed using a micropipet.

The culture was bombarded with tungsten particles coated with the plasmid PBI121 or PBI221.23. Eight microlitres of particle solution was transferred onto a macrocarrier which was previously placed in a holder. The particle/DNA solution was allowed to dry (about one min) before bombardment. The bombardment followed the

Manufacture's instructions. The tissue was bombarded once.

VII. Transient GUS gene expression

Embryogenic suspension cultures of soybean cv. Nattawa, TNS, X2558-13-3-1, and Canatto of similar age were bombarded with plasmid PBI221.23 to determine the efficiency of gene delivery into the suspension culture of these genotypes and the expression of the GUS gene. After bombardment, 100 μ l Ms medium was added into the dish and the dish was sealed with Parafilm. The cultures were incubated for one day and a GUS assay was performed.

VIII. GUS assay

β -Glucuronidase expression was tested by histochemical assay (Jefferson 1988). Bombarded clumps of the suspension cultures were randomly selected at different times after the bombardment and 0.5 ml GUS assay solution was applied and the cells were maintained at 37°C in the dark for one day. The GUS assay solution consisted of 0.2 M sodium phosphate (pH 7.0), 0.1 M $K_3[Fe(CN)_6]$, 0.1 M $K_4[Fe(CN)_6]$, 0.1M EDTA, and 1 mM 5-bromo-4-choro-3-indolyl glucuronic acid (X-gluc) (Clontech Lab. Inc.). The GUS gene expression was assessed by counting and estimating the blue spots in the clumps.

Fig. 4.2. A diagram showing the PDS1000/He bombardment device (modified from Shedden *et al.*, 1995) .

IX. Selection for transgenic clones

Highly embryogenic suspension cultures of cv. Nattawa were then used in the stable transformation study. After bombardment, the cultures were left on the filter paper with 100 μ l Ms medium added overnight before returning to Ms medium in flask. The bombarded cultures were maintained in Ms for two weeks and then transferred to Ms incorporated with kanamycin or hygromycin at 20, 35, or 50 mg/L respectively. Kanamycin and hygromycin were filter-sterilized prior to addition to liquid media. All treatments included the appropriate controls (e.g. the cultures were bombarded with particles without a DNA coating and selected in media containing 20, 35, or 50 mg/L of kanamycin or hygromycin respectively). After about 20 days, the clumps were transferred to fresh Ms media with the same concentrations of kanamycin or hygromycin. Two months later in Ms media, surviving clumps were then selected at higher concentrations of hygromycin: one month at 40 mg/L, and one month at 50 mg/L of hygromycin. Afterwards, the surviving clumps were transferred and maintained in Ms medium. The above experiments were repeated once using the conditions described above.

X. PCR assay

Genomic DNA was extracted as described by Edwards *et al.* (1991) from hygromycin-resistant tissues of suspension culture and control tissues. Two primers

within the GUS gene were chosen to amplify a 610 bp fragment, the 5' primer (GGTGGGAAAGCGCGTTACAAG) located 100 bp downstream and the 3' primer (AGGTGCGGATTACCACTTGC) located 710 bp downstream. The primers were made by Dr. J. Hattori, Plant Research Centre. The thermocycle conditions were as follows: 94°C for 1.5 min, 65° for 1 min, 73° for 1 min for 30 cycles.

XI. Southern hybridization

Genomic DNA was extracted from 250 mg of hygromycin-resistant tissues of suspension culture and control tissues using a G Nome™ DNA isolation kit (Bio 101. Inc.). Genomic DNA was either intact or restricted with HindIII which cleaves PBI221.23 once, just downstream of the 35S promoter of GYH gene. Digested DNA was electrophoresed on a 0.8% agarose gel at 30 V overnight. DNA was denatured by an alkaline treatment and transferred onto a Biotran Plus nylon membrane using 10 x SSC. DNA was fixed to the membrane by baking the membrane at 80° C for 15 min. The membrane was prehybridized for 3-4 hrs using the hybridization without the probe. Hybridization was carried out at 65° C overnight.

The 2.2 kb GUS probe was made by Gene Clean (kit from Promega) by restricting PBI221 with EcoRI and BamHI. The 1 kb HYG probe was made from a BamHI restriction fragment of the PBI221.23 plasmid. The DNA probe was labelled with ³²P-dCTP by using the Prime-a Gene labelling system (Promega).

After hybridization the membrane was washed 2 times at 50 °C for 30 min in 2

x SSC and 0.1% SDS and 2 times at 65 °C for 15 min in 0.1 SSC and 0.5% SDS. The membrane was exposed to X-film at -80 °C for 24 hrs.

Unbombarded soybean suspension culture was used as a control for PCR analysis and Southern hybridization.

XII. Embryo development and germination

Hygromycin-resistant suspension cultures were transferred and maintained on MSBM medium for embryo development. After one month on MSBM medium, developing somatic embryos were separated from each other and transferred to MBO medium for maturation. After one month on MBO medium, the embryos were transferred to an empty Petri dish which was then sealed with two layers of Parafilm. The embryos were partially desiccated for five to seven days in this condition (see Chapter 1) and then returned to fresh MBO medium for germination.

RESULTS

GUS gene expression was equally strong in the suspension cultures of Nattawa, TNS, X2558, and Canatto following bombardment. Over 1000 blue spots could be counted for the tissue of Nattawa from one flask which was divided into three parts and bombarded separately. The suspension culture of Nattawa was selected for the use in the stable transformation study because of its superior embryogenesis response.

Bombardment of Nattawa cultures with PBI121 or PBI221.23 resulted in the similar level of GUS gene expression two days following bombardment (Fig. 4.3). In the case of PBI121, the cultures did not survive the selections containing kanamycin 35 and 50 mg/L. Some of the clumps survived selections containing 20 mg/L kanamycin. However, the GUS assay showed these cultures were negative for the GUS gene. PCR analysis also gave negative results for the NPTII gene. Upon transfer to medium containing 50 mg/L kanamycin, the culture did not survive the higher selection level. Repeated experiments did not produce GUS or PCR positive cultures.

In the case of PBI221.23, there was no survival when the cultures were directly selected at 35 mg/L and 50 mg/L hygromycin. After two months in Ms-20 mg/L hygromycin, a few clumps survived the selection. Some clumps of the controls, however, also survived selection at 20 mg/L hygromycin. These cultures were then selected at higher levels of hygromycin at 40 mg/L and 50 mg/L. A few yellow/green tissues could be seen emerging from brown clumps which were presumed dead. These newly-grown tissues were carefully separated from dead tissues and transferred into fresh

Ms medium lacking hygromycin for proliferation (A5). Controls did not survive higher levels of hygromycin selection. After proliferation for several weeks, a GUS assay was performed. A portion of the culture showed GUS expression (Fig. 4.3). However, some of the clumps, which survived the higher levels of hygromycin selection, did not show GUS expression. The culture was subcultured every month and a sample of tissues was taken for a GUS assay at the same time. However, the portion of the culture expressing the GUS gene in each sample decreased gradually in the following months and no GUS expression was detected after about 10 months. In repeated experiments, a hygromycin-resistant clump (the culture was assigned as A13) was also obtained, but from culture with 35 mg/L hygromycin selection. GUS expression was also lost in this culture.

An expected fragment of 610 bp was amplified from the DNA of A5 and A13 by PCR and showed that the A5 and A13 were positive for the GUS gene (Fig. 4.4).

Integration of foreign DNA in the plant genome was confirmed by Southern hybridization for A5 and A13 in which genomic DNA without restriction was hybridized with the GUS probe (Fig. 4.5). A5 was then divided into two parts: A5-1 and A5-2. After several subcultures, Southern Blot analysis was performed on A5-1, A5-2, and A13 in which genomic DNA was digested with HindIII. Multiple bands were seen for all A5-1, A5-2, and A13, indicating multiple copies of the GUS gene in the plant DNA. A5-1 and A5-2 showed a similar band pattern, suggesting both were from single transformation event. Southern hybridization of the same blot using HYG probe also showed that the HYG gene was incorporated into the plant genome.

Fig. 4.3. Transient GUS gene expression in embryogenic suspension culture two days following bombardment with plasmid PBI121 and PBI221.23.

- a. GUS gene expression in a clump bombarded with plasmid PBI121. 8 x.**
- b. GUS gene expression in clumps bombarded with plasmid PBI221.23. 5 x.**
- c. Clumps bombarded with tungsten particles without DNA coating. 4 x.**
- d. After selection in Ms/Hyg medium, clumps showing strong GUS gene expression. 8 x.**

a

c

After one month on MSBM medium, somatic embryos developed from transformed A13 cultures (Fig. 4.6). The morphology of the embryos appeared normal, as compared to embryos from non-transformed cultures. The embryos were then matured on MBO medium for one month and then partially desiccated in a sealed Petri dish for one week. The desiccated embryos were returned to fresh MBO medium. Upon returned to MBO, embryos started to germinate, cotyledons enlarged, turned green in colour and roots developed. However, after cotyledons died no true leaves developed. Parrott (person. commun.) reported that a long photoperiod of 23 hrs/1 hr dark promoted shoot meristem development. All the procedures, thereafter, were then carried out in a 23-h photoperiod conditions. This time true leaves developed after the cotyledons died. However, the development of the plants stopped after plants grew to 5-10 cm in size. Efforts were also made to recover plants from regular culture which were about the same age but were not subjected to transformation. No plants were recovered from these cultures either.

Fig. 4.4. PCR analysis for surviving cultures. Results indicate that the surviving cultures are positive for the GUS gene. The expected fragment was 610bp.

- 1:** PGEM.
- 2:** H₂O.
- 3:** Blank.
- 4 & 5:** Plasmid negative control, 4: PJD301; 5: PBC17.
- 6 & 7:** Soybean negative control.
- 8 & 9:** Plasmid positive CTL, 8: PBI221, 9: PBI426.
- 10 & 11:** Transformed cultures, 10: A5; 11: A13;
- 12:** λ HindIII.

1 2 3 4 5 6 7 8 9 10 11 12

Fig. 4.5. Southern blot of the hygromycin resistance cultures with the GUS probe. The results indicated that the GUS gene was stably incorporated into the plant genomes.

1:	A5/uncut.
2:	A13/uncut.
3:	Blank.
4:	Control/uncut.
5:	Blank.
6:	A5-1/HindIII.
7:	A5-2/HindIII.
8:	A13/HindIII.
9:	Control.
10:	Blank.
11 & 12:	PBI221.23/BamHI*.
11:	10 pg.
12:	100 pg.

* The size of PBI221.23 is 8 kb, [PUC 19 (2.7 kb) based]. BamHI restriction cuts the plasmid into 1 kb HYG fragment and a 7 kb fragment carrying GUS gene (as shown in the Figure).

1 2 3 4 5 6 7 8 9 10 11 12

23.0-

9.4-

6.6-

4.3-

2.2-

2.0-

Fig. 4.6. Somatic embryogenesis in the transformed culture A13.

- a. Somatic embryos developing normally from an embryogenic clump. 5 x.**
- b. Embryos were separated from each other and transferred onto MBO medium for maturation. 1 x.**
- c. After desiccation, embryos showed vigorous germination. 1 x.**
- d. True leaves developing from germinating embryos. 1 x.**

DISCUSSION

Transformation via microprojectile bombardment is possible, once a highly embryogenic suspension culture is established for a genotype, GUS gene expression was equally strong in the suspension cultures of Nattawa, TNS, X2558, and Canatto following bombardment. In this study, an attempt for stable transformation was carried out for genotype Nattawa only.

Bombarded clumps were left on filter paper overnight before being returned to the medium. This treatment was based on the study of transient expression in which a high level of the GUS expression was obtained following bombardment. The cultures proliferated normally and no obvious death of the cultures occurred when the clumps were returned to the medium after this treatment. Such treatment likely involved in a change in the osmotic condition of the cell, which probably gave rise to a higher frequency of gene delivery and expression of the foreign genes. Changes of cell osmotic pressure have been reported to be correlated with an increase of transformation frequency in maize (Vain *et al.*, 1993).

Bombardment of cv. Nattawa with plasmids PBI121 and PBI221.23 resulted in a similar level of GUS gene expression, indicating that both plasmids were delivered with equal effectiveness into the plant cells. However, the NPTII gene is under the control of the NOS promoter and the HYG gene is driven by 35S promoter in two plasmids, respectively. The 35S promoter is a strong promoter in plant cells and might activate stronger expression of the HYG gene, which in turn may have given cells a better chance

of surviving exposure to hygromycin. On the other hand, kanamycin has been suspected to interfere with some plant cells *in vitro* (Pezzotti *et al.*, 1991; Kuehnle and Sugli, 1992). Bommineni *et al.* (1993) reported that selection with kanamycin at early stages of embryo culture caused inhibition in their development. Kanamycin interference may have been detrimental for the survival and amplification of bombarded and transformed cells for soybean. Sato *et al.* (1993) reported transformation of soybean, cv. Fayette, with the NPTII gene, but the selective agent used was geneticin (G418 sulphate). Different marker-based selective agents, therefore, could have different effectivenesses for selection (Dennehey, *et al.*, 1994).

In the early stages after selection, only a portion of the surviving culture showed positive GUS responses. Since direct addition of the GUS assay solution to the culture is toxic to plant cells, it is difficult to separate GUS positive and negative clumps. Clones which were sectored for GUS activity were also reported by Finer and McMullen (1991). The reason of gradual disappearance of GUS activity is unknown. One possible explanation could be the silencing of the transgene, a phenomenon often documented in the literature (Parrott *et al.*, 1994). Methylation of the GUS gene may also be a possibility (Amasino *et al.*, 1984; John and Amasino 1989; Chen *et al.*, 1994).

Southern blot analysis indicated stable incorporation of the foreign genes. A5-1 and A5-2 cultures showed similar band patterns, indicating a single transformation event. A13 also showed multiple bands, suggesting multiple transformation events, or a single transformation with multiple copies in the plant genome. The transformation efficiency appeared comparable to that reported by Finer and McMullen (1991) in which an average

of 3 independent transgenic lines were obtained per bombarded flask of an embryogenic suspension culture. In this study, the bombarded culture was divided into three parts and subjected to three selection conditions respectively. The stable transformation was obtained from only one condition. This condition was probably more favourable and effective for the selection. In general, the transformation efficiency in soybean remains low. Parrott *et al.* (1994) bombarded 150 to 200 embryogenic clumps of each of three soybean genotypes and obtained only one transgenic line.

The two lines generated were selected at either 20 or 35 mg/L hygromycin. These lines later tolerated hygromycin at 50 and 60 mg/L. Direct selection at 50 mg/L hygromycin did not give rise to survival for bombarded cultures. Since the transformation was low, it is difficult to compare the efficiency of different selection strategies. Since both lines generated were selected at lower hygromycin concentrations, it may indicate that step-wise selection is more effective to establish stable transformed cultures under the conditions used. However, direct selection at higher levels (50 mg/L hygromycin) has also been also reported (Finer and McMullen 1991). Different bombardment conditions can lead to different degrees of damage to the cells, which in turn likely results in different frequencies of stable transformation (Sanford *et al.*, 1994). Also different types of particle guns can result in different degrees of damage to the cells (Finer *et al.* 1992; Hunold *et al.*, 1994). Step-wise selection probably provides a better chance for cells to survive.

The lack of plant recovery from the somatic embryos was probably due to the old age of the cultures. The embryos developed from the cultures which were over three

years old, including pre-bombardment and post-bombardment time. Difficulties were also encountered in regenerating plants from the control cultures which were the same age but were not subjected to bombardment and hygromycin selection. Somaclonal variation may be the reason. Parrott *et al* (1994) reported that recovered transgenic soybean plants were aneuploids with an additional 5-10 chromosomes.

Stable transformation results obtained here indicate that long-day-photoperiod-adapted or daylight-insensitive soybean germplasm types are amenable to transformation via bombardment of somatic embryogenic suspension cultures and extends the previous reports of transformation via bombardment of embryogenic suspension cultures in other types of soybean germplasms. Bombardment of newly established cultures will likely to lead to the recovery of transformed lines into plants.

CHAPTER 5

DEVELOPMENT OF DESICCATION TOLERANCE IN A TRANSFORMED SOYBEAN EMBRYOGENIC LINE

INTRODUCTION

Preservation of transformed embryogenic lines and somatic embryos would broaden the use and distribution of genetic engineering technology.

Valuable embryogenic cultures can be preserved by two general ways; namely, cryopreservation and artificial seed. While cryopreservation uses extremely low temperature to preserve the culture, artificial seed relies on the more natural process of partial desiccation to conserve living structures.

Tolerance to dehydration in most higher plants is restricted to the seeds. *In vitro*-cultured embryos generally lack such a property, but tolerance can be induced by a number of treatments (Anandarajah and McKersie, 1990). ABA is mostly used for such induction. This chemical has been used successfully to induce desiccation tolerance in embryos derived from various sources, including somatic embryos in alfalfa (McKersie *et al.*, 1989; Senaratna *et al.*, 1989, 1990; Brown 1994) and carrot (Iida *et al.*, 1992), microspore-derived embryos in *Brassica* (Senaratna *et al.*, 1991; Takahata *et al.*, 1992; Brown *et al.*, 1993) and zygotic embryos in wheat (Brown, 1994). Induction can be at various stages during embryo development, depending on the species and culture protocols, such as in liquid suspension culture (Iida *et al.*, 1992), or on solid medium (Senaratna *et al.*, 1990; Brown 1994).

To date, there is no report on study of artificial seeds on soybean somatic embryos, especially in transformed embryogenic lines of this plant. In this study, ABA induction and desiccation tolerance was investigated in the transformed soybean

embryogenic line (A13), and somatic embryos developed from it.

MATERIALS AND METHODS

The transformed embryogenic line A13, described in Chapter 4, was used in this set of experiments. The culture was maintained by subculturing with fresh Ms medium monthly. Embryos developed normally from this culture on MSBM medium.

ABA ($\pm$ *cis-trans* ABA, 99+ % purity, Sigma Chemical Co.) stock was made by dissolving the chemical in a few drops of KOH and then diluted in distilled water. The solution was filter sterilized and stored in the dark at -20°C .

ABA was applied to the culture at different times and stages of embryo development to investigate potential of induction of tolerance to desiccation; namely, globular stage (proliferation) in liquid suspension culture, the embryo developmental stage in MSBM medium, and the maturation stage in MBO medium. The ABA concentrations used in this study ranged from $1\mu\text{M}$ to $100\mu\text{M}$. This range has been used successfully to induce desiccation tolerance in alfalfa somatic embryos (Senaratna *et al.*, 1990; Brown 1994), *Brassica* microspore-derived embryos, wheat zygotic embryos (Brown, 1994), and carrot somatic embryos (Iida *et al.*, 1992). An embryogenic clump usually consisted of about 50 globular-staged somatic embryos for this line. At least one clump or embryos developed from one clump was used in each treatment. Survival was measured as regrowth of the embryos and estimated as percent of the embryos used in a treatment.

I. Embryogenic suspension culture

ABA was added into regular Ms medium at concentrations of 1, 10, 50, and 100 μM at the time of subculture. Ten days, 20 days, and 30 days after transfer to ABA-containing medium, the embryogenic clumps were sampled and transferred to an empty 30 x 60 mm Petri dish in which a sterile filter paper was placed and 130 μl sterile distilled water was previously added. The application of water was to prevent quick dehydration of the embryos. The clumps were carefully separated into smaller pieces. The embryogenic clumps were then subjected to a desiccation process. The desiccation process was as follows: A Petri dish which contained culture tissues was placed in a desiccator for approximately 24 hrs at 25 $^{\circ}\text{C}$ in dark and transferred daily through a series of desiccators in which the relative humidity was progressively lower from 97% to 43% (Brown *et al.*, 1993). The relative humidity in each desiccator was controlled by the use of different saturated solutions (Table 5-1), based on Winston and Bates (1960) and Weast (1977). Two humidity indicators (measuring RH) were placed into each desiccator to ensure the RH reached its level before the dishes were transferred to the next one. After desiccation, embryogenic clumps were transferred to MSBM medium.

II. Embryo development

The suspension culture was maintained in Ms medium. The culture was then

treated with ABA in MSBM medium at two times during embryo development.

i) The early stage of embryo development

Embryogenic clumps were transferred to MSBM medium containing 1, 10, 50, 100 μ M ABA. Filter-sterilized ABA was added to MSBM medium at about 40-50 °C after the medium had been autoclaved. Embryos were maintained in the MSBM/ABA media for 15 days and then subjected to desiccation as described before. After desiccation, the embryos were returned to fresh MSBM medium.

ii) The later stage of embryo development

Embryogenic clumps were first allowed to develop on ABA-free MSBM medium for 15 days. After 15 days, the embryos developed to the torpedo to cotyledonary stage with a size of 2-4 mm. The embryos were then transferred to MSBM/ABA medium at the same ABA concentrations and maintained in this medium for another 15 days. After ABA treatment, the embryos were subjected to the desiccation procedure and then returned to fresh MSBM medium.

III. Embryo maturation

Embryos were allowed to develop on regular MSBM medium for one month.

Developed embryos were then transferred to MBO/ABA media and maintained for one month. ABA-treated embryos were desiccated and then returned to fresh MBO medium.

The survival of the embryos after desiccation was recorded. The survival was measured as the tissue growth of the embryos.

A diagram illustrating these protocols is shown in Fig. 5.1.

IV. Determination of water content of matured embryos during desiccation

In one experiment, the water content in embryos at different stages during desiccation was determined. The fresh weight of matured embryos was first determined. The embryos were then subjected to the desiccation procedure. The weight of the embryos were determined after treatment in each desiccator. After in desiccator # 6, the embryos were dried in an oven at 80 °C for 24 hrs and the dry weight of the embryos was then recorded. The percent water content of the embryos at different stages of desiccation was calculated as follows:

$$\text{Percent water content} = (W_1 - W_2)/W_1 \times 100$$

W_1 : Weight of desiccated embryos

W_2 : Weight of dry embryos

V. Tolerance of embryos to different relative humidities

Sensitivity and survival of somatic embryos at different stages to different RH levels was also determined. Somatic embryos at different stages, namely globular embryos in suspension culture, developing embryos in MSBM medium, and matured embryos, were each divided into four groups. Group 1 (D3) was desiccated in desiccator #3 for five days. Group 2 (D4) was desiccated in desiccator #3 for one day and in desiccator #4 for four days. Group 3 (D5) was desiccated in desiccators #3 and #4 for one day each and then in desiccator #5 for three days. Group 4 (D6) was desiccated in desiccators #3, #4, and #5 for one day each and then in desiccator #6 for two days. After five days in desiccation, the embryos were transferred to appropriate media. The illustration showing these experiments was shown in Fig. 5.5.

Fig. 5.1. A flow-chart showing the protocol for ABA treatment and desiccation in the soybean transformed embryogenic line A13.

- 1 Cotyledons in MSB medium for embryo induction.
- 2 Establishment of embryogenic suspension culture.
- 3 Selection of bombarded culture with hygromycin.
- 4 Maintenance of transformed cultures.
- 5 Embryo development.
- 6 Embryo maturation.
- 7 ABA treatments.
- 8 Desiccation.
- 9 Recovery of desiccated cultures.

Table 5-1. The relative humidity used in each desiccator.

Desiccator	Saturated Compound	Theoretical % Relative Humidity ¹
1	K_2SO_4	97
2	Na_2CO_3	87
3	$NaCl$	75
4	NH_4NO_3	63
5	$Ca(NO_3)_2 \cdot 4H_2O$	51
6	$K_2CO_3 \cdot 2H_2O$	43

¹. Values based on Winston and Bates (1960) and Weast (1977).

RESULTS

Addition of ABA to Ms medium did not appear to affect the growth of suspension cultures. The cultured cells proliferated normally in ABA-containing media. The morphology of the culture containing ABA at different concentrations appeared normal compared to the control. After one month, when the medium usually started to turn turbid or cloudy and needed to be subcultured, medium containing a higher concentration of ABA, ie. 50 or 100 μ M, appeared still clear. But the media became cloudy a few days later. This implies that ABA may decrease metabolism activity slightly. Upon transfer to MSBM medium, embryos developed normally from ABA-treated clumps. A slightly higher percent of normal embryos was observed from the cultures which had been treated with ABA in Ms at 1 μ M and 10 μ M (not shown).

No noticeable effect was observed for embryo development in MSMB medium containing ABA. However, in the later stage of embryo development on MSBM and during maturation on MBO medium, embryos appeared to lose their green colour and turned light yellow in colour in ABA-containing media. Such loss of green colour was even more obvious when embryos were exposed to ABA for longer times and at higher concentrations.

With none of the stages tested, namely, globular proliferation stage in suspension culture, embryo development stage and maturation stage, did ABA show an effect on induction of tolerance to desiccation (Fig. 5.2). However, untreated embryos exhibited different degrees of tolerance to desiccation at different stages. Embryos at the globular

stage in suspension culture showed some degree of survival. However, such ability was completely lost during embryo development on MSBM medium and no embryos survived the desiccation when the embryos developed for two weeks on MSBM medium (2-4 mm in size) (Fig. 5.3). By thirteen days on MSBM medium, about 25% of the embryos survived the desiccation. Such tolerance to desiccation was further increased when embryos were matured on MBO medium. By 30 days on the maturation medium (60 days after transfer from the suspension culture), around 65% embryos showed tolerance after exposure and equilibration in 43% RH for at least three days (Fig. 5.2.5.3 & 5.6). The percent water content of matured embryos at different stages during the desiccation procedure was also determined. After desiccation, matured embryos contained only about 7% water (Fig. 5.4).

In one experiment, embryos were matured on MBO medium for about 50 days when some of the embryos exhibited different degrees of germination, indicated by dark-green cotyledons. Such embryos showed a poor survival (< 10%) after being subjected to desiccation.

Different-staged embryos also showed different degrees of desiccation tolerance when the embryos were treated under 75%, 63%, 51% and 43% RH respectively. The survival of globular-staged embryos showed an gradual increase if desiccated at higher RHs, such as at 51%, 63%, and 75%. (Fig 5.5). Matured embryos also showed an increased survival if treated at higher RH. Developing embryos, however, were extremely sensitive to desiccation treatments. Treatments of developing embryos at higher RHs up to 75% did not show an obvious increase of the survival. Less than 10%

of the embryos survived even they were desiccated under a condition of RH 75% for five days (Fig. 5.5).

Fig. 5.2. Percent survival of ABA-treated soybean embryos at different stages of development. The embryos were treated with ABA with different concentrations (1, 10, 50, and 100 μ M) and were progressively moved to RH 43%. The value is the average of at least 2 replicates.

Day 0. The embryos were treated in Ms/ABA media for 10 days prior to desiccation.

Day 15. The suspension-cultured embryos were transferred to and maintained on MSBM/ABA media for 15 days. After 15 days, developing embryos (2-4 mm in size) were subjected to desiccation.

Day 30. Embryos were first allowed to develop on MSBM medium. After 15 days, developing embryos were transferred and maintained on MSBM/ABA media for another 15 days. The ABA treated embryos were then subjected to desiccation.

Day 60. Embryos were developed on MSBM medium for 30 days. Developed embryos were then transferred and matured on MBO/ABA media for 30 days. Afterwards the embryos were subjected to desiccation.

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PM-1 3 1/2" x 4" PHOTOGRAPHIC MICROCOPY TARGET
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PRECISIONSM RESOLUTION TARGETS

Fig. 5.3. Desiccation tolerance of soybean somatic embryos during embryo development and maturation over a period of 60 days. These embryos were not treated with ABA but progressively desiccated to RH 43%. Vertical bar indicates the standard error of the mean of at least three replicates.

Fig. 5.4. Percent water content of matured somatic embryos at different stages during the desiccation treatment. Vertical bar indicates the standard error of the mean of at least 4 replicates. See Table 5.1 for the respective RH of each desiccator.

Fig. 5.5. Tolerance of soybean embryos of different stages of development to different desiccation treatments. The value is the average of two replicates.

Suspension. Suspension-cultured globular embryos.

Developing. Developing embryos of 2-4 mm in size.

Maturation. Matured embryos.

RH 75 Embryos were desiccated in desiccator #3 for five days.

RH 63 Embryos were desiccated in desiccator #3 for 1 day and transferred maintained in desiccator #4 for 4 days.

RH 51 Embryos were desiccated in desiccator #3 for 1 day, #4 for 1 day and then transferred and maintained in desiccator #5 for 3 days.

RH 43 Embryos were desiccated in desiccator #3, #4, and #5 for 1 day each and then transferred and maintained in desiccator #6 for 2 days.

Fig. 5.6. Photos showing the survival of the embryos at different stages after desiccation. Embryos were gradually desiccated to a RH of 43% as described in the text, and then transferred back to the appropriate media.

- a. Embryogenic clumps (globular stage-d embryo) in suspension culture. 1 x.
- b. Developing embryos on MSBM medium. 1 x.
- c. Developed embryos on MSBM medium. 1 x.
- d. Matured embryos. 1 x.

DISCUSSION

Unlike in alfalfa, *Brassica* and carrot, ABA did not show an induction of tolerance to desiccation in soybean somatic embryos at all stages of development. But at certain stages, the embryos developed tolerance to the dehydration treatment without ABA induction.

Globular-staged embryos showed some survival to desiccation. These embryos were developmentally stable in that they never developed into the next stage of development (i.e. torpedo or cotyledonary-staged embryos) in the suspension culture. Such developmental stability may be helpful in the tolerance to dehydration treatment. However, upon transfer to MSBM medium where the embryos started to increase in size and were developing to the cotyledonary stage, the tolerance to desiccation was completely lost. The developing embryos were extremely sensitive to desiccation treatments; over 90% of the embryos could not survive desiccator #3 for five days (RH 75%). This indicates that the embryos were either not tolerant to the dehydration treatment or depletion of nutrients (media) during desiccation was very detrimental.

The matured embryos could tolerate a dry environment as low as 43% RH. This is comparable to the results reported for ABA-induced tolerance in alfalfa, *Brassica*, and wheat embryos (Senaratna *et al.*, 1990; Brown 1994). In a study of soybean embryo germination, Bailey *et al.* (1994) reported that a portion of mature somatic embryos could even survive a dry environment as low as 33% RH for five days. As reported in other species (Senaratna *et al.*, 1990; Brown, 1994) the somatic embryos have to reach

certain size and developmental stage before they could tolerate desiccation. Development of desiccation tolerance in mature zygotic embryonic axes of soybean has also been recorded (Sun and Leopold, 1993). The highest desiccation tolerance was at the stage when the embryos had been matured on MBO medium for 30 days. The majority, i. e. 65%, of the embryos survived desiccation. Embryos grown from a clump did not have a similar size or stage of development. When fully developed and high quality embryos were selected, survival was increased to above 85% (not shown). Longer maturation times were not beneficial and when embryos showed signs of germination on MBO medium, they were unable to survive desiccation. This is in accordance with the observation in alfalfa in which embryos beginning to germinate lost ability to tolerate a drying treatment (Senaratna *et al.*, 1990).

The cause of induction of desiccation tolerance is not known. The embryos were developed on MSBM medium containing 6% maltose and matured in MBO medium containing 4% sucrose. The osmolarities in these media were relatively high. Buchheim *et al.* (1988) documented that the moisture content of soybean somatic embryos decreased gradually in a high osmolarity medium. Gradual reduction of water content in embryos may induce the development of desiccation tolerance. On the other hand, the increased osmolarity has been associated with elevation of endogenous ABA levels (Morris *et al.*, 1988, Anandarajah and McKersie, 1990) which may in turn have induced desiccation tolerance. The replacement of ABA with a high concentration of sucrose was successful in inducing desiccation tolerance in alfalfa somatic embryos (Anandarajah and McKersie, 1990). In addition, induction of desiccation tolerance by low temperature, water,

nutrient, and heat stress have also been reported (Senaratna *et al.*, 1989, 1990). Matured embryos exhibit a higher desiccation tolerance. Buchheim *et al.* (1988) reported that the length, fresh weight, and dry weight of somatic embryos of soybean, induced directly from cotyledons, increased during maturation. Accumulation of sugars in the embryos may increase the stability of the membranes in the dry state (Crowe *et al.*, 1984).

The development of desiccation tolerance may also be induced by endogenous ABA. The developing soybean embryos accumulate large quantities of ABA during the first three weeks of embryogenesis (Quebedeaux *et al.*, 1976; Ackerson, 1984). During this period of time the embryo develops from globular to cotyledonary stage. Senaratna *et al.* (1990) reported that application of ABA at the torpedo and cotyledonary stage could induce desiccation tolerance in alfalfa somatic embryos. In a study of ABA-induced desiccation tolerance in *Brassica napus* microspore-derived embryos, Pomeroy *et al.* (1994) reported that addition of ABA into the medium increased endogenous ABA levels by approximately 20 times. Thus, the endogenous ABA induced by addition of ABA in medium may play a role in induction of desiccation tolerance in this species. If soybean somatic embryos have a high level of endogenous ABA, addition of ABA probably would not show an observable function. A high level of endogenous ABA in soybean somatic embryos has been suggested in a number of studies. In contrast to alfalfa and *Brassica*, somatic embryos of soybean are known to have difficulty in germinating without a desiccation treatment. The inability of germination in immature zygotic embryos has been correlated with a high concentration of endogenous ABA in the embryos (Rajasekaran *et al.* 1982; Prevost and LePage-Degivty 1985). Desiccation

of somatic embryos may cause a decrease in the endogenous ABA level (Buchheim *et al.*, 1989). If the above is true, the high level of endogenous ABA may have played a role in the induction of tolerance to desiccation in soybean somatic embryos.

This study demonstrated development of desiccation tolerance in a soybean transformed line. Further optimization of this approach should facilitate the research on artificial seeds and preservation of transformed soybean lines as well as lead to more efficient recovery of somatic embryos into plants.

SUMMARY AND GENERAL CONCLUSIONS

Somatic embryogenesis of long-day-photoperiod-adapted soybean was systematically investigated and characterized. The results support the hypothesis that the LDPA soybean had a different capacity for somatic embryogenesis - LDPA soybean showed a higher capacity to form somatic embryos compared to SDPA soybean. These germplasm types appeared to be more responsive to somatic embryogenesis compared to ordinary soybean. The differential responses of somatic embryogenesis discovered in the study provides an alternative way to induce a large number of somatic embryos in suspension culture for some genotypes which are of agronomic importance but did not show promising signs for somatic embryogenesis using immature zygotic embryo explants. The establishment and characterization of the efficient somatic embryogenesis system provides a base for many studies including both pure and applied research for LDPA soybean.

A new Particle Bombardment Device was characterized and found to deliver gene into cultured alfalfa and soybean cells efficiently, as compared to a commercial bombardment device. The results are in accordance with the hypothesis that the new particle bombardment device can deliver foreign genes into alfalfa and soybean cells as efficiently as a commercial device. The new device also has other advantages, such as a low operating cost, less damage to cells, operating simplicity, and it is easy to build. These properties provides the potential to engineer crops using the bombardment technique more efficiently and reduce the costs for genetic engineering. The major disadvantage of the device is that at higher bombardment pressure the sample can be dislodged.

Various aspects of alfalfa transformation via microprojectile bombardment using somatic embryogenesis were systematically studied and characterized. The results indicated that the biolistic approach for plant transformation is not just a simple procedure of physical gene delivery. Many biological factors are also important for the transformation using this approach. Proper manipulation of the cultures could increase the potential of development of a transformation system as well as the transformation efficiency. The results strongly support the hypothesis that development stages of somatic embryogenesis in alfalfa affect transformation following microprojectile bombardment and a biolistic approach will circumvent the host-range limitation of *Agrobacterium*-mediated approach in alfalfa transformation. The information obtained in the study provides a solid foundation leading to the establishment of an efficient transformation system for alfalfa using a biolistic approach.

Transformation of LDPA soybean via the biolistic approach was investigated. A transformation system via this approach using somatic embryogenesis in a suspension culture was developed for these germplasm types. The hypothesis that LDPA soybean could be transformed by via a biolistic approach using somatic embryogenesis system was thus supported by the results. Although the mature plants were not recovered from transformed small plantlets, the major reason for this has been identified. The use of newly-developed cultures as transformation targets will likely overcome the regeneration problem. On the basis of the transformation system developed, LDPA soybean could be transformed by agronomically important genes.

Desiccation tolerance in transformed soybean somatic embryos was characterized.

The hypothesis proposed that ABA treatment induced desiccation tolerance in a transformed soybean line was not supported by the study, since ABA was not effective in inducing desiccation tolerance during any stage of embryo development. However, somatic embryos, after maturation, developed tolerance to desiccation without the use of externally-applied ABA. A high level of endogenous ABA in matured somatic embryos was proposed to have induced desiccation tolerance in soybean. This study is the first one to characterize artificial seed technology in transformed somatic embryos. The method characterized and developed allows for more efficient embryo recovery and provides the potential to preserve transformed somatic embryos in soybean.

Future direction:

- i) Increase of the induction frequency of somatic embryogenesis from immature cotyledon explants and bombardment of cotyledons may provide an alternative approach for transformation of soybean, especially for the varieties in which an embryogenic suspension culture is difficult to establish.
- ii) Reduction of the time for establishment of embryogenic suspension cultures and use of younger suspension cultures as bombardment targets may lead to recovery of fertile plants from transformed cultures.
- iii) Modification of culture media and protocols could improve and increase the efficiency to preserve transformed soybean cultures by artificial seed technology.
- vi) Alfalfa petioles and stems at certain *in vitro* stages (i.e. day 5 in B5h medium) may provide a superior target for bombardment transformation. Somatic embryos also appear to be a superior target for bombardment.

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APPENDIX I

PARTICLE AND DNA PREPARATION FOR PDS1000/HE

I. Particle preparation

- Weigh 60 mg tungsten particles in a 1.5 ml microfuge tube
- Add 1 ml 100% EtOH
- Vortex 1 min
- Centrifuge 1 min at maximum setting
- Remove as much EtOH as possible
- Add 1 ml sterile distilled H₂O
- Vortex 1 min
- Centrifuge 1 min at maximum setting
- Remove as much H₂O as possible
- Wash with water again as above
- Resuspend the particle in 1 ml sterile glycerol solution
- Distribute in 50 μ l aliquot into 1.5 ml microfuge tube while in vortexing

II. DNA preparation

- 50 μ l tungsten particles
- 5 μ l DNA (1 μ g/ μ l)
- 50 μ l 2.5M CaCl_2
- 15 μ l 0.1 M spermidine
- Vortex for 2 min
- Allow particles to precipitate by sitting the tube on bench for a 5-10 min
- Remove supernatant
- Wash with 250 μ l 100% EtOH, resuspend particles by briefly vortexing
- Centrifuge and remove supernatant
- Wash with EtOH again (optional)
- Resuspend particles in 40 μ l 100% EtOH. The solution is for 4-6 bombardments

APPENDIX II

PARTICLE AND DNA PREPARATION FOR THE NEW PARTICLE BOMBARDMENT DEVICE

I. Particle preparation

- Weigh 100 mg tungsten particles in a 1.5 ml microfuge tube
- Add 1 ml EtOH%
- Vortex 1 min
- Centrifuge 1 min at maximum setting
- Remove as much EtOH as possible
- Add 1 ml sterile distilled H₂O
- Vortex 1 min
- Centrifuge 1 min at maximum setting
- Remove as much H₂O as possible
- Wash with water again as above
- Resuspend the particle in 1 ml sterile distilled H₂O
- Distribute in 25 μ l aliquot into 0.5 μ l microfuge tube while in vortexing

II. DNA preparation

- **25 μ l tungsten particles**
- **5 μ l DNA (1 μ g/ μ l or less as 0.5 μ g/ μ l), mix**
- **25 μ l 2.5 M CaCl_2 , mix**
- **10 μ l 0.1 M spermidine, mix**
- **Allow particles to precipitate by sitting the tube on bench for 5 min**
- **Remove 50 μ l supernatant. The remaining solution is for 3 bombardments**
- **Load 3 μ l solution on metal screen in syringe for bombardment**

APPENDIX III**COMPOSITION OF THE BUFFER FOR GUS ASSAY**

CHEMICAL	CONCENTRATION
$\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ (pH 7.0)	0.2 M
$\text{K}_3[\text{Fe}(\text{CN})_6]$	0.1 M
$\text{K}_4[\text{Fe}(\text{CN})_6]$	0.1 M
EDTA	0.1 M
X-Gluc.	0.001 M

APPENDIX IV**MEDIA FOR SOYBEAN SOMATIC EMBRYOGENESIS****I. MSB: Embryo induction medium.**

Composition	Chemicals	Concentrations
Salt	MS (Murashige & Skoog's)	-
Vitamin	B5 (Gamborg's)	-
Growth Regulator	2,4-D	180 μ M
Carbohydrate	Sucrose	1.5%
Other	-	-
Gel Agent	Bactoagar	0.8%
pH = 5.8	-	-

II. Ms: Suspension culture medium.

Composition	Chemicals	Concentrations
Salt	MS (Murashige & Skoog's)	-
Vitamin	B5 (Gamborg's)	-
Growth Regulator	2,4-D	22.5 μ M
Carbohydrate	Sucrose	6%
Other	Glutamine	15 mM
Gel Agent	-	-
pH =5.7	-	-

III. MSBM: Embryo development medium.

Composition	Chemicals	Concentrations
Salt	MS (Murashige & Skoog's)	-
Vitamin	B5 (Gamborg's)	-
Growth Regulator	-	-
Carbohydrate	Maltose	6%
Other	Activated Charcoal	0.5%
Gel Agent	Gelrite	0.25%
pH = 5.8	-	-

IV. MBO: Embryo maturation and germination medium.

Composition	Chemicals	Concentrations
Salt	MS (Murashige & Skoog's)	-
Vitamin	B5 (Gamborg's)	-
Growth Regulator	-	-
Carbohydrate	Sucrose	4%
Other	-	-
Gel Agent	Gelrite	0.25%
pH = 5.8	-	-

APPENDIX V

MEDIA FOR ALFALFA SOMATIC EMBRYOGENESIS

I. B5h: Embryo induction medium (two-step and three-step protocols).

Composition	Chemicals	Concentrations
Salt	B5 (Gamborg's)	-
Vitamin	B5 (Gamborg's)	-
Growth Regulator	2,4-D	4.5 μM
	Kinetin	0.9 μM
Carbohydrate	Sucrose	2%
Other	Glutamine	5.5 mM
	Glutathione	32.5 μM
	Serine	95.1 μM
	Adenine	7.4 μM
	CaCl ₂ ·2H ₂ O	5.1 mM
Gel Agent	Bactoagar	0.9%
pH = 5.5	-	-

II. SH4K: Embryo induction medium (Two-step protocol).

Composition	Chemicals	Concentrations
Salt	SH (Schenk & Hildebrandt's)	-
Vitamin	SH (Schenk & Hildebrandt's)	-
Growth Regulator	2,4-D	4.5 μ M
	Kinetin	0.9 μ M
Carbohydrate	Sucrose	1%
Other	Proline	25 mM
	Thioproline	0.4 mM
	Potassium	50 mM
Gel Agent	Gelrite	0.25%
pH = 5.5	-	-

III. B5mod: Suspension culture medium.

Composition	Chemicals	Concentrations
Salt	B5 (Gamborg's)	-
Vitamin	B5 (Gamborg's)	-
Growth Regulator	2,4-D	4.5 μ M
	NAA	0.5 μ M
Carbohydrate	Sucrose	1.5%
Other	-	-
Gel Agent	-	-
pH =5.5	-	-

IV. BOi2Y: Embryo development medium.

Composition	Chemicals	Concentrations
Salt	Blaydes (Blaydes's)	-
Vitamin	Blaydes (Blaydes's)	-
Growth Regulator	-	-
Carbohydrate	Sucrose	3 %
Other	Yeast extract	0.2%
	Myo-inositol	100 mg/L
Gel Agent	Bactoagar	1%
pH = 5.5	-	-

V. 1/2 MSO: Embryo germination medium.

Composition	Chemicals	Concentrations
Salt	1/2 MS (Murashige & Skoog's)	-
Vitamin	1/2 MS (Murashige & Skoog's)	-
Growth Regulator	-	-
Carbohydrate	Sucrose	3%
Other	-	-
Gel Agent	Gelrite	0.25%
pH =5.8	-	-

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