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I am submitting herewith a thesis written by Jennifer Lea Nash entitled "Long Term Production of Genetically Transformed Somatic Embryos of Orchardgrass (*Dactylis glomerata*) in Suspension Culture." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree Master of Science, with a major in Plant and Soil Science.

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LONG TERM PRODUCTION OF GENETICALLY TRANSFORMED SOMATIC EMBRYOS OF ORCHARDGRASS (*Dactylis glomerata)* IN SUSPENSION CULTURE

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Abstract

Orchardgrass is the only Gramineae species whose somatic embryos progress to a fully mature (germinable) stage within one liquid medium. As embryos mature, the scutellar epidermis begins to dedifferentiate and cells are sloughed off. These cells divide and form new embryos creating a cyclic regeneration system. Genetic transformation of these cells provides the opportunity to mass produce transformed embryos. In this study, somatic embryos removed from leaves were transformed via microprojectile bombardment with a gene construct containing both the *uid*A gene coding for expression of the GUS protein and the *bar* gene which inactivates phosphinothricin (PPT), the active ingredient in herbicides such as Basta and bialaphos. Both genes were driven with the *ubi*1 promoter. Embryos were cultured before bombardment on Schenk and Hildebrandt medium amended with 30 µM dicamba (SH-30) and containing 0.3 M each of mannitol and sorbitol for a 24 h pre and 24 h post bombardment osmotic treatment. The embryos were then transferred to fresh SH-30 medium and cultured in the dark at 21^{0}C for 2 wk to form callus. Samples of the resulting calli were incubated in 5-bromo-4-chloro-3-indolyl β-D glucuronic acid (x-gluc) substrate to test for GUS expression. All embryos whose callus showed GUS expression were placed into suspension culture. The cultures were sieved through a 710 µm mesh screen at 30 d intervals and the medium and cells which passed through were returned to culture. From the tissue that was

removed, 100 embryos were incubated in x-gluc to determine the number of transformed embryos present. The number of embryos expressing GUS was found to increase more than 2 fold between 30 d and 60 d after culture initiation and did not decrease until 120 d after initiation. One hundred remaining embryos were plated on SH medium without dicamba (SH-0) and incubated in 16 h light/8 h dark at $21^0C/15^0C$ in order to germinate. Regenerated plants were potted and kept in the greenhouse. A young leaf of each plant was brushed with a 0.1% solution of Basta to test for tolerance to the herbicide. A total of 143 plants were tested. Six displayed complete tolerance and 10 showed a localized reaction where the herbicide was applied. Of the six tolerant plants, one was confirmed by Southern blot analysis to contain the *bar* transgene.

Six cultures remaining from the above experiment were each sieved through a 710 μm and a 210 μm screen to create three fractions of different cell size. Each fraction was then split into two cultures. One culture was suspended in liquid SH-30 medium ammended with 12 mM each of proline and serine plus 2.5 mg/L bialaphos added as a selective agent. The other culture was suspended in SH-30 medium with casein hydrolysate and used both as a control and to continue the culture. The control cultures were then split into a casein hydrolysate control and a control containing 12 mM each of proline and serine. At 14 d intervals, fresh weight of each culture was measured and viable cells were counted in 1 ml samples of each culture. At

49 d after culture initiation, all cultures were incubated in x-gluc to test for both the number of embryos expressing GUS and the extent to which GUS was expressed. It was observed that, although more embryos exhibited some amount of GUS expression in non-selective cultures, cultures which had undergone selection pressure displayed uniform GUS expression throughout the tissue.

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Chapter One

Introduction

Orchardgrass (*Dactylis glomerata L.*) is a cool season forage grass grown in temperate regions of the world, especially in the Eastern United States, for pasture and hay (Christie and McElroy, 1995). A specific genotype, 'Embryogen-P', developed and released by Conger and Hanning (1991), has been used in basic tissue culture investigations (Conger et al., 1986; 1988). This genotype possesses regeneration systems that are unique and among the most efficient and repeatable in the family Gramineae. These include the initiation and development of somatic embryos directly from mesophyll cells in cultured leaf segments (Hanning and Conger, 1982; Conger et al., 1983; Trigiano et al., 1989) and the full development (to a germinable stage) of somatic embryos directly in suspension (liquid) culture (Gray et al., 1984; Gray and Conger, 1985; Conger et al., 1989). The leaf culture system has been recently utilized in gene transfer experiments (Denchev et al., 1995; Conger et al., 1996).

Suspension cultures have been employed in plant genetic transformation experiments involving both Angiospermae and Gymnospermae (see Literature Review for details and specific references). Among forage and turf grass species, Hartman et al. (1994) used particle bombardment to transform suspension culture cells of three creeping bentgrass (*Agrostis palustris* Huds.) cultivars with a gene for tolerance to the

herbicide Herbiace™. Southern hybridization confirmed the presence of the transgene in these plants. Spangenberg et al. (1995b) obtained transgenic plants of tall and red fescue (*Festuca arundinacea* Schreb. and *F. rubra* L.) after bombardment of suspension cultures with a plasmid coding for hygromycin phosphotransferase (*hph*) activity. Perennial ryegrass (*Lolium perenne* L.) suspension cultures were also transformed with the *hph* gene (Spangenberg et al.,1995c).

In some cases, cells have been returned to suspension after gene transfer with DNA coated microprojectiles (Finer and McMullen, 1991; Hagio et al., 1991; Sato et al., 1993; Bommineni et al., 1993; 1994). Although this is often done as a method of introducing selection pressure, some experiments were designed to study the length of time that transformed cells could remain in suspension. For example, Bommineni et al., (1993; 1994) transformed embryos of white (*Picea glauca* Moench.) and black (*Picea mariana* Mill.) spruce with the β-glucuronidase (*uid*A) gene which produces a blue precipitate (GUS expression) when exposed to the chemical substrate 5-bromo-4-chloro-3-indolyl β-D glucuronic acid (x-gluc). In both species the number of embryos expressing GUS declined after 35 d in suspension.

As mentioned above, genetic transformation experiments are currently being conducted with the orchardgrass leaf culture system. These involve bombardment with tungsten microprojectiles coated with the DNA plasmid pAHC25 (Christensen and Quail, 1996). This plasmid possesses the *uid*A

and *bar* (tolerance to phosphinothricin based herbicides) genes. A homemade particle inflow gun (PIG) based on the design by Finer et al. (1992) is used to propel the microprojectiles.

Prior to the present study, the orchardgrass suspension culture system had not been used in transformation experiments. However, because embryos will fully develop within a single liquid medium, the system presents experimental opportunities not available with other grass or cereal species. As the embryos mature, cells are sloughed from the scutellum. These cells are capable of dividing and developing into new embryos which in turn will repeat the process as they mature (Gray et al., 1984). Therefore it represents a cyclic system of potential infinite totipotency (Conger et al., 1989).

The present study involved initiating suspension cultures from somatic embryos which had been bombarded with the pAHC25 plasmid. These cultures were then sampled at 30 d intervals to study the effects of suspension culturing on transformed cells. The objectives were to determine: (1) if transformed embryos, when placed into suspension culture, would produce new transformed embryos, (2) if the number of transformed embryos produced would increase with time in culture, and (3) the length of time transformed embryos would continue to be produced.

Chapter Two

Literature Review

Somatic Embryogenesis and Embryogenic Suspension Cultures

The first reported observations of in vitro somatic embryogenesis were from cultures of carrot tissue (*Daucus carota* L.) by Reinert, (1958) and Steward et al., (1958a,b). Norstog (1970) succeeded in inducing embryo-like structures from young barley (*Hordeum vulgare* L.) zygotic embryos. He observed the growth of a coleoptile leaf from some of these structures. This was the first observed occurrence of possible somatic embryogenesis in a cereal species. Later, Thomas et al. (1977) derived embryo-like structures and regenerated shoots from cultured zygotic embryos of sorghum (*Sorghum bicolor* L.). The new structures arose from the scutellum of the original cultured embryos. It was not until the early 1980's, however, that true somatic embryogenesis was documented in cereals (refer to reviews by Vasil and Vasil, 1986; Morrish et al., 1987; Vasil, 1987).

Conger et al. (1983) demonstrated somatic embryogenesis from orchardgrass leaf tissue. This was significant because the embryos initiated and developed directly from single mesophyll cells without an intermediate callus phase. Sections from the base of the innermost (youngest) two leaves of tillers were cut into 3 to 4 mm length segments and plated on Schenk and Hildebrandt (1972) medium amended with 30 μM dicamba (SH-30). A

gradient response was observed in which segments closest to the basal end produced embryogenic callus and those more distal produced less callus and more direct embryos. These embryos were capable of germinating and producing plants.

Later, Gray et al. (1984) established orchardgrass suspension cultures in liquid SH-30 medium with and without the addition of 1.5 g/L of casein hydrolysate, a combination of several amino acids derived from bovine milk. They found that cultures maintained in medium without casein hydrolysate produced cell masses which would divide to form root primordia but not embryos within the culture. In cultures which contained casein hydrolysate, the cell masses divided to form embryogenic cell masses and developing embryos with almost no root primordia. Developing embryos in suspension culture then produced callus which formed additional embryos and embryogenic cell masses. By placing somatic embryos and embryogenic callus in liquid SH-30 medium with 3 g/L casein hydrolysate embryos developed to a completely mature (germinable) stage within the same medium. Embryos could be germinated and plantlets grown to maturity in soil. This is the first and only known case of somatic embryos developing to a germinable stage within one liquid medium in a gramineous species.

Experiments were also conducted to determine the optimum levels of medium components for proliferation of orchardgrass suspension cultures. These included a study of the effects of dicamba and casein hydrolysate

(Gray and Conger, 1985), combinations of proline and serine (Trigiano and Conger, 1987), and proline tested alone and in combination with glycine, histidine, serine and threonine (Trigiano et al., 1992). No amino acid alone induced the production of embryos; however, combinations of proline plus serine and proline plus threonine did stimulate production. The combination of 12.5 mM each of proline and serine was the most effective. Ammonium ion (NH_4) as ammonium sulfate was then tested both alone and with proline/serine combinations (Trigiano et al., 1992). Although embryo production was stimulated more by NH_4 than by the proline/serine combination, medium supplemented with NH_4 caused abnormal embryo formation. Lower germination/regeneration rates were also found in medium with NH_4 supplementation than with the amino acids alone. Addition of serine to medium containing NH_4 caused a decrease in embryo formation which could be reversed with the addition of proline, but the culture never achieved the proliferation rate of NH_4 alone.

The single cell origin of somatic embryos in orchardgrass suspension culture was shown by Conger et al. (1989). Beginning with a culture of individual cells and small cell masses, it was observed that embryos formed by differentiating from these cells and masses 5 wk after culture initiation. These embryos, once mature, then underwent a proliferation and dedifferentiation of their scutellum cells which were sloughed off to produce new embryos (Conger et al., 1989). Therefore, the system is theoretically

both perpetual and totipotent. Through histological studies, it was further shown that there is little difference between somatic embryos in suspension culture and zygotic embryos in mature caryopses.

Transformation

Genetic transformation is a useful procedure for incorporating genes into plants that cannot receive them through sexual crosses. Many methods have been developed to produce transgenic plants; thus far the most repeatable and successful methods are infection with *Agrobacterium* (*tumefaciens* or *rhizogenes*), direct DNA uptake by protoplasts and microprojectile bombardment.

Agrobacterium is a plant pathogen which is able to transfer a piece of its Ti (tumor inducing) plasmid (referred to as the T-DNA) into the plant which it infects. The natural purpose of this procedure is to cause increased cell division within the plant (known as crown gall disease) thus producing copies of the bacteria's DNA. It is possible to remove a portion of the T-DNA from the plasmid and replace it with a desirable gene for transformation (Fraley et al., 1986). The remaining DNA allows for the plant to be infected, but the transgene, instead of the disease gene, is incorporated into the plant genome. Successful transformation with *Agrobacterium* depends on many factors, among these are the strain and concentration of the bacterium and

the length of time that the plant tissue and bacteria are incubated together. This is a successful method for dicotyledonous species, especially in the family Solonaceae (Visser et al., 1989; Atkinson and Gardner, 1991; Newell et al., 1991; Figueira-Filho et al., 1994; Kumar et al., 1995). Several forage legumes have also been transformed with this method including white clover, *Trifolium repens* L., (White and Greenwood, 1987; Voisey et al., 1994) and alfalfa (*Medicago* sp.; Deak et al., 1986; Shahin et al., 1986; Loesch-Fries et al., 1987; Chabaud et al., 1988; D'Halluin et al., 1990; Damani and Arcioni, 1991; Ninkovic et al., 1995).

Despite the above successes, *Agrobacterium* has generally not been effective for transforming monocotyledonous species because of their apparent resistance to this type of infection (Christou, 1995). Recent experiments with rice, *Oryza sativa* L., (Hiei et al., 1994) and maize, *Zea mays* L., (Ishida et al., 1996) however, have shown successful transformation by this method. This research group reported that careful selection of all parameters is essential for successful transformation.

An alternate method for transformation is direct DNA uptake by protoplasts (plant cells whose walls have been removed through enzymatic digestion). Polyethylene glycol, PEG, (Krens et al., 1982) and electroporation (Fromm et al., 1985) have been used to facilitate the uptake of DNA by the cells. Successful transformation by DNA uptake with protoplasts is usually low due to poor regeneration of whole plants from

these cells. Several factors influence regeneration including plant species and genotype and the conditions under which the protoplasts are cultured. In general, plant regeneration from protoplasts of gramineous species has been difficult (Potrykus, 1990). The first monocot transformed via DNA uptake by protoplasts was maize (Rhodes et al., 1988) with a gene for neomycin phosphotransferase (NPTII) which codes for resistance to the antibiotic kanamycin. Mature plants were obtained and tested for the NPTII gene by Southern hybridization analysis (Southern, 1975) which confirmed its presence. None of the regenerated plants produced viable pollen and only one produced silks. No seed was obtained after outcrossing.

DNA uptake by protoplasts has been used in many experiments to transform grasses. Potrykus et al. (1985) transformed Italian ryegrass (*Lolium multiflorum* Lam.) protoplasts with the amino glycoside phosphotransferase [APH(3′)II] gene. Transformation of colonies was confirmed with Southern hybridization analysis and enzyme assays. Orchardgrass was transformed with an amino glycoside phosphotransferase type IV (APH IV) hygromycin resistance gene by Horn et al. (1988). This was the first report of obtaining transgenic plants in a forage grass. From three experiments on cultures containing 1.5×10^6 to 2.6×10^6 protoplasts/ml, 95 resistant callus lines were obtained and Southern analysis confirmed the presence of the APH IV gene. Control and transformed callus lines produced 90 regenerated plants, some of which were confirmed by Southern analysis

to contain the foreign gene. During the past five years, much work has also been conducted with fescue species. Wang et al. (1992) transformed tall fescue protoplasts with the *hph* and the *bar* genes. The *bar* gene confers resistance to the herbicides Basta™ and bialaphos which increase ammonia levels within plant cells causing death. Plantlets were regenerated from the protoplasts and analyzed with Southern hybridization to confirm transformation.

The microprojectile bombardment method is both easier and more efficient than *Agrobacterium* or direct DNA uptake in cereals and grasses. This method consists of propelling DNA coated microscopic particles, usually gold or tungsten, into target tissue via a high pressure explosive charge. Many methods have been used to produce this charge: gunpowder (Klein et al., 1987; 1988; Sanford et al., 1987; 1988), electrical discharge (Christou et al., 1988;1991) and compressed gasses (Oard et al., 1990; Sautter et al., 1991). Among the first cereal species successfully transformed via microprojectile bombardment were maize (Fromm et al., 1990; Gordon-Kamm et al., 1990) and rice (Christou et al., 1991). A low cost and easy to construct particle inflow gun (PIG) was developed later and uses a blast of helium to propel the particles through a vacuum chamber (Finer et al., 1992; Vain et al., 1993a). A PIG was used in the experiments described in this thesis.

The parameters of the particle gun, including vacuum pressure, target distance and helium pressure vary and must be determined for each system.

However, generalizations can be made on certain factors such as particle size, plasmid size and cell type. Kausch et al. (1995) bombarded maize suspension cells to study the effects of small versus large particles penetrating the tissue. They found that particles less than or equal to 1 μm in diameter did not usually penetrate past the first cell layer. Larger particles (2.0 μm to 3.5 μm) were found to travel further into the tissue, but no particles of any size penetrated deeper than the first few cell layers. One of the most important parameters, and perhaps the most difficult to control, is the tissue itself. Cell type, age and location as well as other factors may all affect a cell's ability to be transformed. Some research suggests that many cells are capable of being either transformed or regenerated, but not both (Potrykus, 1990; Laparra et al., 1995). Because successful transformation requires plant regeneration, it is important to consider the tissues being used in experiments.

Microprojectile bombardment has been used to successfully transform several cereal species. Wan et al. (1995) transformed anther derived callus of maize with the *bar* and *uid*A genes. Plants were regenerated and Southern hybridization analysis confirmed the integration of the transgenes in both callus and plants. Immature barley embryos were transformed with the NPTII and the *uid*A genes (Ritala et al., 1995). Selection was conducted at the rooting stage of plant regeneration and those plants that survived were analyzed by Southern hybridization analysis which confirmed integration of

the genes. Casas et al. (1993) used immature sorghum embryos in transformation experiments with *bar* and *uid*A. Callus lines were produced from bialaphos tolerant embryos. Plants were regenerated from six independent callus lines; their leaves were tolerant to application of 0.6% Basta and Southern hybridization analysis confirmed integration. Weeks et al. (1993) regenerated transgenic wheat (*Triticum aestivum* L.) plants, but only one had normal fertility. Further examples of genetic transformation in cereals by microprojectile bombardment can be found in recent reviews by Morrish et al. (1993), McElroy and Brettell (1994) and Mendel and Teeri (1995).

Forage and turf grasses transformed via microprojectile bombardment include creeping bentgrass (Zhong et al., 1993), tall and red fescues (Spangenberg et al., 1995b) and orchardgrass (Conger et al., 1993; Denchev et al., 1995). Conger et al. (1993) transformed orchardgrass suspension cultures and leaf segments with two different bombardment devices, the Biolistics™ PDS 1000 helium gun (Sanford et al., 1991) and the PIG (Finer et al., 1992). Transgenic tissues were obtained after bombardment with both devices. Results indicated no difference between the commercial and the lab-built guns. A comparison was made by Denchev et al. (1995) between plasmids containing both the *uid*A and the *bar* genes under the control of either the CaMV35S or the *ubi*1 promoters. The *ubi*1 promoter produced 2.3 times more GUS spots than the CaMV35S promoter. The GUS spots were

also larger and more intense with *ubi*1 than with CaMV35S. Somatic embryos were produced from bombarded leaves and these embryos also expressed GUS. Therefore, *ubi*1 appears to be a more efficient promoter for orchardgrass than the commonly used CaMV35S. Taylor et al. (1993) obtained similar results after bombarding cell suspensions of several grass species with plasmids containing the maize *Adh*1 or *ubi*1 promoter. The *ubi*1 promoter resulted in more transient GUS expression than the *Adh*1 promoter In all species. More recently, Denchev et al. (1996) obtained regenerated transformed plants from somatic embryogenesis of bombarded orchardgrass leaf tissue indicating that these tissues are competent for both transformation and regeneration.

Transformation of Suspension Cultures

Finer and McMullen (1991) suggested that the best target tissues may be embryogenic cultures due to the regenerative capabilities of these tissues. They based this idea on experiments conducted with soybean suspension cultures that were transformed with the *hph* gene. After bombardment, the tissues were resuspended in selection medium containing hygromycin and cultured for an additional 3 to 4 months. During this period, embryos were periodically removed in order to conduct Southern hybridization analysis on both embryos and the plants regenerated from them. Twelve different

transgenic clones were obtained and all were confirmed by Southern analysis to contain the *hph* gene.

Suspension cultures of many plant species have been transformed with particle bombardment including herbaceous dicots (Finer et al., 1991; Russell et al., 1992; Sato et al., 1993), woody species (Wilde et al., 1992; Bommineni et al., 1993; 1994), cereals (Mendel et al., 1989; Gordon-Kamm et al., 1990; Hagio et al., 1991; Cao et al., 1992; Ritala et al., 1993; Vain et al., 1993b; Kausch et al., 1995) and grasses (Taylor et al., 1993; Hartman et al., 1994; Spangenberg et al., 1995a; 1995b).

In studies on tobacco, Russell et al. (1992) tested various bombardment parameters and it was observed that those which had the most effect on transformation were strength of the promoter, the use of a helium device instead of gun powder propelled particles and the addition of reagents such as mannitol and sorbitol to the medium before bombardment (osmotic treatment) to reduce turgor pressure within the tissue. Vain et al.(1993b) also observed the advantage of adding an osmotic treatment to the bombardment medium. Plating maize suspension cultures on medium containing 0.2 M each of mannitol and sorbitol for 4 h pre and 16 h post bombardment resulted in a 2.7 fold increase in expression of the transient *uid*A gene and as much as a 6.8 fold increase in stable transformation for bialaphos and glufosinate resistance.

Microprojectile bombardment has been used in transformation experiments with both Angiospermae and Gymnospermae. Wilde et al. (1992) transformed embryogenic suspensions of yellow-poplar (*Liriodendron tulipifera* L.) with *uid*A and NPT II and through somatic embryogenesis obtained regenerated plants that expressed both genes. Bommineni et al. (1993; 1994) placed bombarded embryos of both white and black spruce into suspension culture. White spruce tissues continued to express GUS after 8 months and gene transfer was confirmed by Southern hybridization analysis. In black spruce, embryos showed high levels of GUS 2 d after bombardment but after 35 d in culture, GUS expression was no longer observed.

In tall fescue, Kuai and Morris (1995) also found a negative relationship between the length of time that transformed tissue was in suspension culture and GUS expression. They observed a peak expression period of approximately 10 wk, after which expression began to decline. After 1 yr in culture there was almost no GUS expression which could be visually detected. They consider this to be due to the developmental stage of the cells and not the age of the culture.

In experiments with cereals, Mendel et al. (1989) found that smaller plasmids (5.1 kb) were more effective than large plasmids (12.8 kb and 14.2 kb) in transforming barley tissues, presumably because they are more stable. Barley suspension cultures were transformed using both gun powder and helium bombardment devices (Ritala et al., 1993). Helium bombardment

was superior, producing an average of 7000 GUS spots per g of tissue, while gun powder bombardment produced an average of 2000 GUS spots per g of tissue. It was also determined that the best time for transformation was when the suspension cultures were in an accelerated growth phase. Cao et al. (1992) discovered that ammonia levels increased as much as 16 fold 1 d after herbicide exposure in 15 cm tall nontransformed rice plants sprayed with 1% Basta and only two fold in plants transformed with the *bar* gene. After this initial increase, ammonia levels in transformed plants decreased to levels present before herbicide application while levels in nontransformed plants remained high.

Suspension culture cells of three creeping bentgrass cultivars were transformed with a gene for tolerance to the herbicide Herbiace™ (Hartman et al., 1994). From five experiments of 8 to 14 filter paper disks covered with suspension culture tissue, 271 plants were obtained that were tolerant to 1.5 mg/ml of the herbicide were obtained. The tolerant plants were then tested with a concentration of 2 mg/ml and 55 tolerant plants were recovered from two of the original tested cultivars. Southern hybridization confirmed the presence of the transgene in these plants.

Tall and red fescue suspension cultures were bombarded by Spangenberg et al. (1995b) with a plasmid coding for the *hph gene*. The cells were subjected to selection in liquid or on solid medium after bombardment. An approximate two fold increase of transgenic cells was

found in liquid compared to solid medium in both species. Gene transfer was confirmed by Southern and northern analyses and by the hygromycin phosphotransferase enzyme assay. Spangenberg et al. (1995c) also transformed perennial ryegrass suspension cultures with the *hph* gene. The suspension cells were placed on filter paper disks and plated onto solid medium in culture dishes. A total of 145 dishes of embryos were bombarded. Embryos from 49 of the dishes were plated onto solid medium and the remainder were placed into liquid medium, each containing hygromycin as a selection factor. Seven tolerant calli were obtained from the cells on solid medium and 36 tolerant calli were retrieved from the liquid medium. Plants were obtained from these calli and tested with both Southern and northern hybridization analyses and integration of the foreign *hgh* gene into the genome of the plants was confirmed.

Although many transformation experiments have been conducted with monocot suspension cultures, these cells been have been placed back into culture in only a few cases (Finer and McMullen, 1991; Hagio et al., 1991; Sato et al., 1993; Bommineni et al., 1993; 1994). This thesis presents the results of experiments in which bombarded somatic embryos of orchardgrass were placed into suspension culture and the effects of this culturing were studied over time.

Chapter Three

Materials and Methods

General Procedures

1. Tissue Culture

Culturing of Leaves for Somatic Embryos

Basal leaf segments measuring 3 cm long were taken from the two innermost leaves of Embryogen-P tillers (Hanning and Conger, 1982; Conger et. al., 1983). The leaves were split along the midvein and sterilized in 2.6% sodium hypochlorite for 2 min followed by rinsing in three changes of sterile water. They were then cut into segments of approximately 3 to 5 mm^2 and plated on Schenk and Hildebrandt (1972) medium amended with 30 mM 3,6-dichloro-2-methoxybenzoic acid (dicamba). This medium is hereafter referred to as SH-30. Incubation was in the dark at 21^0C for approximately 21 to 28 d to allow for embryo formation.

Suspension Culturing of Somatic Embryos

Somatic embryos were plated on solid SH-30 and incubated in the dark at 21^0C for 14 d to allow for callus formation. Embryos and callus were then removed from the solid medium and placed into 125 ml erlenmeyer flasks containing 15 ml liquid SH-30 medium amended with 3 g/L casein hydrolysate. These flasks were placed on a shaker at 100 rpm at ambient-

temperature where they remained during the course of the experiments (up to 180 d).

2. Transformation

Plasmid Description and Isolation

The plasmid used was pAHC25 (Figure 1) developed by Christensen and Quail (1996). This plasmid contains the *uid*A reporter and *bar* selectable marker genes. As mentioned, the *uid*A gene codes for β-glucuronidase which produces a blue precipitate when exposed to the chemical substrate 5-bromo-4-chloro-3-indolyl β-D glucuronic acid (x-gluc). The *bar* gene codes for phosphinothricin acetyl transferase (PAT) which inactivates phosphinothricin (PPT), the active ingredient of herbicides such as Basta™, glufosinate, bialaphos and Herbiace™. Both genes were driven by their own copy of the maize ubiquitin promoter (*ubi*1).

Bacterial cultures containing the pAHC25 plasmid were plated on solid Luria broth (LB) medium containing the antibiotic ampicillin (100 mg/L) as a selection agent and incubated in the dark at 37^0C for 16 h. Individual colonies were selected and placed into 5 ml of liquid LB medium with ampicillin in 50 ml screw top tubes. The tubes were incubated under the same conditions as above on a shaker at 200 rpm until the cells reached the log phase of growth (approximately 7 h). The 5 ml cultures were then placed into 100 ml of the same medium and incubated under the same conditions

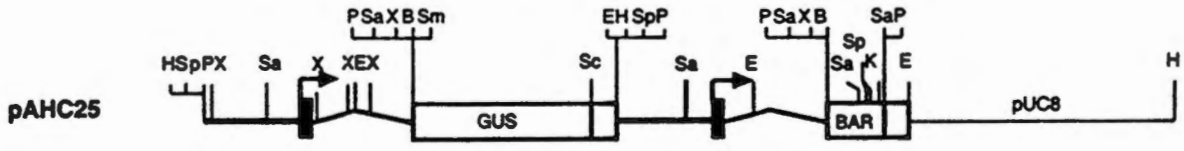


Figure 1: Linear map of the pAHC25 plasmid developed by Christensen and Quail (1996) and used in this study.

until the cells again reached log phase (approximately 16 h). Plasmid isolation was accomplished with the Qiagen plasmid maxi prep protocol (Crowe, 1992) using alkaline lysis and a resin binding column.

Particle Preparation

Three mg of tungsten particles (1.1 mm diameter; Bio Rad, Richmond, CA) were coated with 12 ml of pAHC25 plasmid DNA (1 mg/ml). The particles were then mixed with 40 ml of 2 M $CaCl_2$ and 20 ml of 5 M spermidine. After centrifugation, the pellet was washed in 140 µl 70% ethanol and the particles were resuspended in 40 ml of absolute ethanol.

Bombardment

Somatic embryos were removed from incubated leaf sections (discussed above) and placed 60 to a plate on solid SH-30 medium containing an osmotic treatment of 0.3 M each of mannitol and sorbitol. They were cultured on this medium for 24 h pre and 24 h post-bombardment. The tissues were placed in the microprojectile gun (Finer et. al., 1992) bombardment chamber at a distance of 19 cm from the syringe filter (Figure 2). A 500 µm screen was placed above the embryos at 16 cm from the syringe in order to disperse the particles over the surface of the tissue. A vacuum pressure of 597 mm Hg was pulled in the chamber, and a helium pressure of 552 x 10^3 Pa (80 PSI) was used to propel the particles. Target tissues were bombarded twice with 6 ml of particle suspension to increase the number of transformation events.

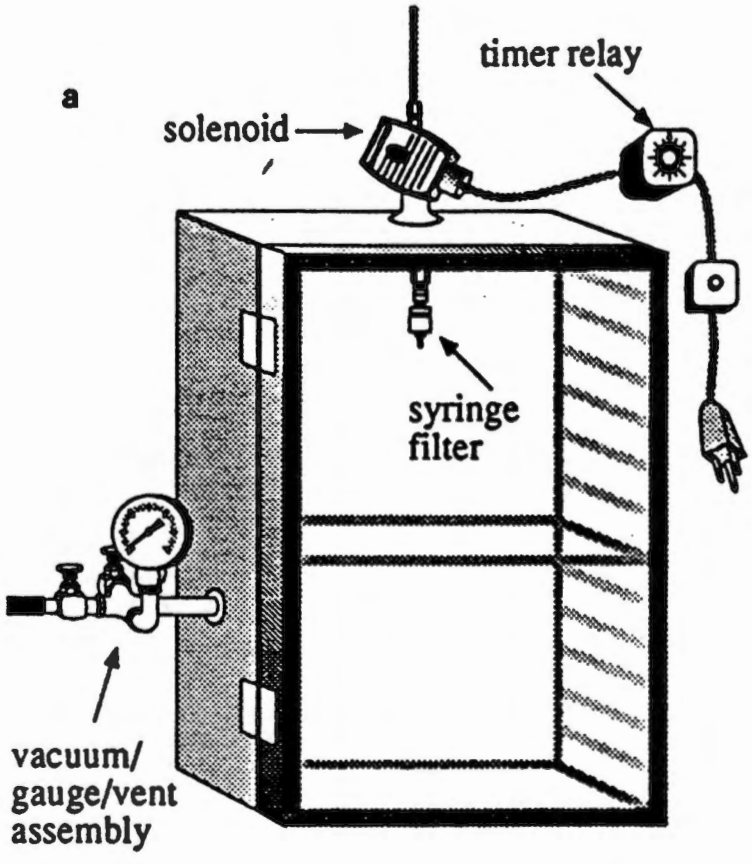


Figure 2: Particle inflow gun illustration from Finer et al. (1992). Parameters used with the gun were 552 x 10^3 Pa helium pressure and 597 mm Hg vacuum pressure with the target tissue placed 19 cm from the tip of the syringe filter and a 500 µm mesh screen above the tissue at 16 cm from the tip of the syringe filter.

Testing for Transformation

GUS expression was determined by placing the bombarded tissues in x-gluc and vacuum infiltrating the substrate into them. The tissues were left in the x-gluc and incubated in the dark at 37^{0}C for 16 h. Blue spots on the tissue were then counted with the aid of a stereomicroscope.

Regenerated plants were tested for tolerance to Basta™ when they had reached the 3 to 4 tiller growth stage. A 3 cm section in the center of an inner leaf of each plant was brushed on both sides with a 0.1% solution of the herbicide. After 4 d, the tillers were examined for herbicide reaction in the form of tissue necrosis.

3. Molecular Verification of Transformation

Southern Hybridization Analysis

Young leaf tissue (approximately 1 g) was removed from plants and placed immediately into liquid nitrogen. Samples were then ground to a powder with a pestle in a chilled mortar . Tannins and phenols released from the tissue were bound to polyvinyl-polypyrrolidone (PVP) which was added during the grinding process. DNA was then extracted using a Puregene kit from Gentra Systems, Inc. (Research Triangle Park, NC).

Twenty mg of DNA from each sample were digested for 16 h with the *pst* 1 restriction enzyme (New England Biolabs, Beverly, MA). An additional 2.5 ml of *pst*1 enzyme was then added and the DNA digested for another 7 h.

A sample of the pAHC25 plasmid was also digested for 1 h with the *pst*1 enzyme. Tracking dye was added to all samples and they were heated for 5 min in a 65^{0}C water bath. A 0.7% agarose gel was prepared in an HE 99X maxi submarine agarose unit (Pharmacia Biotech, Alameda, CA). DNA from each of the six experimental plants and the two controls were loaded into two separate lanes each (16 lanes total). In addition, plasmid DNA and a lambda DNA/*Hin*d III fragments molecular weight marker (Life Technologies, Inc., Gaithersburg, MD) were loaded into one lane each. All lanes contained 25 ml of sample. The gel was then run at 20 v for 18 h and stained for 5 min in enough 0.5 mg/ml ethidium bromide to cover the gel.

Gels were placed in a plastic, acid proof container, covered with 0.25 M HCl and placed on an orbital shaker for 20 min at 50 rpm. The HCl was then poured out; each gel was rinsed with distilled water and placed into denaturation buffer for 30 min. The buffer was removed and the gel rinsed and incubated two times for 15 min each in neutralization buffer. The DNA was transfered to Hybond™-N membrane (Amersham Life Science, Buckinghamshire, England) via an overnight (approximately 18 to 20 h) capillary blot procedure. The membrane was removed from the blot, marked and then dried for 20 min on clean chromatography paper (Whatman Ltd, Maidstone, England) in a laminair flow hood. The membrane was then placed face down on the transilluminator ultraviolet light source (UVP, Upland, CA) for 7 min to bind the DNA to the membrane.

The blots were placed in clear plastic heat sealable bags (Kapak®/Scotchpak heat sealable pouches; Kapak® Corp., Minneapolis, MN) with 20 ml of DNA hybridization solution (Puregene kit, Gentra Systems, Inc., Research Triangle Park, NC). A 25 µl sample of a 0.6 kb fragment of DNA was obtained from a *pst*1 digest of the pAHC25 plasmid. The plasmid DNA was denatured by boiling for 5 min and used as a binding template to form a probe for the *bar* gene. From the RadPrime DNA labeling system (Life Technologies, Gaithersburg, MD), 5 ml of [a-^{32}P] dCTP (approximately 50 mCi), and 1 ml each of 10mM dATP, dGTP and dTTP was added to the template DNA. Twenty ml 2.5X random primer solution and 21 ml of sterile distilled water were added to this solution. Klenow fragment (1 µl), a reactive enzyme, was then added, mixed, and incubated for 30 min in a 37^{0}C water bath. The probe solution was then boiled for 5 min, cooled on ice and 50 µl was added to the plastic bag containing the membrane. Air bubbles were removed from the liquid and the bag was heat sealed.

The membrane was incubated overnight (approximately 18 h to 20 h) at 65^0 C. It was then removed from the hybridization bag and washed first in 0.2X DNA hybridization solution for 20 min and then in a 0.1X solution for 20 min at 65^{0}C. The membrane was washed in the 0.1X solution several times to remove any unbound radioactive probe. It was then wrapped in clear plastic wrap, placed in an x-ray film cassette with a piece of x-ray film and exposed overnight (18 h to 20 h) at -80^{0}C. The film was developed using

GBX developer and fixative (Kodak; Rochester, NY) to show the hybridization pattern.

Procedures for Specific Experiments

1. Optimization of Bombardment Parameters

Experiments were conducted to test the effects of various bombardment parameters on GUS expression in target tissues. The parameters tested were: (1) distance from the syringe filter (16 cm or 17.5 cm) of a 500 μm mesh screen placed above the target tissue, (2) helium pressure of 414 x 10^3 Pa or 552 x 10^3 Pa (60 PSI or 80 PSI) and (3) vacuum pressure (445 mm Hg or 597 mm Hg) pulled within the chamber. Leaves were prepared as for producing somatic embryos but were not cut into 3 to 5 mm segments. Each petri dish contained four leaf halves from four different leaves and the corresponding halves of the same leaves were plated in a second dish. There were three replications for each combination in a completely randomized design with a 2 x 2 x 2 factorial arrangement of treatments. The factorial arrangement was incomplete with two treatments missing. Both treatments incorporated 17.5 cm screen distance and 552 x 10^3 Pa helium pressure, with one treatment using 445 mm Hg and the other using 597 mm Hg vacuum pressure. These treatments were not included based on unpublished data suggesting that these combinations

would not have a significant effect on transformation. Statistical analysis was performed according to Schlotzhauer and Littell (1987).

2. Comparison of DNA Annealing Procedures

Two protocols were compared for annealing plasmid DNA to microprojectiles. The first, which had previously been used in our lab, was described above under "Particle Preparation" and came from Hunold et al. (1994). The second (Perl, et al., 1992) used 50 µl of 1 M $Ca(NO_3)_2$ to anneal the DNA. A total of five replications of two leaf halves were bombarded and tested for GUS expression 24 h post bombardment.

3. Osmotic Treatment Comparisons

Equimolar amounts of mannitol and sorbitol ranging from 0.0 M to 1.0 M at 0.1 M intervals were added to SH-30 medium and 30 embryos were plated on each. Embryos remained on the medium 24 h before and 24 h after bombardment. Particle preparation and bombardment procedures for each plate were as previously described. The greatest increase in GUS expression was obtained with 0.3 M of each osmoticum. An experiment was then conducted to compare the 0.3 M treatment to the control (0.0 M) to ensure that there was a statistically significant difference. Petri dishes with the two media and embryos were prepared as described above. There were four replications for each medium.

4. Transformation of Somatic Embryos and Initiation of Suspension Cultures

Somatic embryos at various developmental stages were taken from incubated leaf segments. Embryos were plated, 60 per dish, in a 1.5 cm diameter area in each of four 100 x 15 mm petri dishes per experiment. Bombardment was as described and after the osmoticum post-treatment, the embryos were incubated on solid SH-30 medium to form callus. After 15 d, a sample of callus from each embryo was removed and tested for transient GUS expression. All embryos and remnant callus whose samples showed any blue stain were then placed into suspension culture in SH-30 medium amended with 3 g/L casein hydrolysate.

5. Testing Suspension Culture Embryos for Transformation

Each culture was sieved through a 710 µm mesh screen at 30 d intervals. The liquid which passed through the screen contained embryogenic cells and was returned to culture. The embryos removed from culture were used to test for transformation. One hundred randomly selected embryos from each flask were tested for GUS expression. An additional 100 embryos were plated on SH medium without dicamba (SH-0) and incubated in 16 h light (fluorescent bulbs with an intensity of 80 µmol $m^{-2}s^{-1}$)/8 h dark at 21^{0}C/ 15^{0}C to germinate. When the plantlets possessed roots 2 cm or longer, they were planted in peat pellets (Jiffy-7; Jiffy Products (N.B.) Ltd.,

Shippagan, Canada) and maintained in clear plastic self-sealing bags under the incubation conditions mentioned above. When the plantlets had reached a height of approximately 8-10 cm, they were placed in soil-less potting mix (Fafard Growing Mix; Conrad Fafard, Inc., Agawam, MA) and transferred to the greenhouse. Once the plants in the greenhouse were of sufficient age (3 to 4 tillers) they were tested for Basta™ tolerance as described.

6. Selection Testing within Suspension Cultures

Previously initiated suspension cultures consisting of five suspected transformed cultures and one control, all of varying ages, were used to test the effect of adding bialaphos to the liquid medium as a selection agent so that, theoretically, only cells transformed with the *bar* gene would survive and produce new embryos. Each culture was used to initiate three cultures consisting of varying tissue mass. This was accomplished by sieving the cultures through a 710 μm and a 210 μm mesh screen to obtain three fractions of varying tissue size. Fraction one consisted of tissue that did not pass through the 710 μm screen, fraction two contained tissue that passed through the 710 μm screen but not the 210 μm screen, and all remaining tissue that passed through the smaller screen was placed into fraction three.

Liquid medium containing 12.5 mM each of proline and serine was prepared according to Trigiano et al. (1992). Bialaphos at 2.5 mg/L was added to this medium. The amount of bialaphos used was based on

unpublished results of leaf culture experiments performed in our laboratory by Conger and Denchev (1994). Each fraction of cells was then divided and an equal weight of cell mass (fresh) was placed into two suspension cultures, one containing 3 g/L casein hydrolysate to be used as a control and to continue culture growth, and one containing 12.5 mM each of proline and serine plus 2.5 mg/L bialaphos as a selection agent. At 14 d intervals, each fraction initiated culture was poured into a 50 ml Oak Ridge tube and centrifuged at 1000 g (3000 rpm) for 1 min. The liquid was removed from the tubes and fresh weights were determined. After data collection, each culture was resuspended in fresh medium. A 1 ml sample was taken from each fraction culture and 1 to 2 drops were placed on microscope slides. The cells were stained with diacetylfluorescein to test for viability. One thousand cells were counted from each sample to determine the percentage of viable cells.

After the first 14 d interval, the control cultures in casein hydrolysate medium had reached a sufficient mass to allow division into two different control media. One medium contained 12.5 mM each proline and serine and was used as a control for the bialaphos treatment. The other control medium contained 3 g/L casein hydrolysate and was included so that both media containing proline and serine could be compared to the medium commonly used for orchardgrass experiments in our laboratory and also used in the other experiments of this study.

Chapter Four

Results

Optimization of Bombardment Parameters

Experiments were conducted to determine the optimum parameters for bombarding leaf segments. The parameters studied were helium pressure, vacuum pressure and distance of 500 µm screens placed above the tissue. A vacuum pressure of 597 mm Hg produced a much higher number of GUS spots than 445 mm Hg (Table 1). An analysis of variance (ANOVA) showed this to be the only factor that was statistically significant (Table 2).

Comparison of DNA Annealing Procedures

In tests of DNA annealing protocols, the $CaCl_2$ plus spermidine treatment produced more spots per leaf than did the $Ca(NO_3)_2$ treatment (Table 3); however, the difference ($P>0.05$) was not statistically significant (Table 4). The $CaCl_2$ plus spermidine protocol was used in all subsequent experiments.

Table 1: Optimization of bombardment protocols. Leaf segments were placed 19 cm from the syringe filter of the microprojectile gun and a 500 μm mesh screen was placed over the tissue at 16 or 17.5 cm from the filter. Vacuum pressures of 445 mm Hg or 597 mm Hg and helium pressures of 414×10^3 or 552×10^3 Pa were tested to determine the optimum conditions for transformation. The leaves were incubated in x-gluc and GUS expressing spots were counted. Data are presented as the average of three replications.

Helium Pressure (Pa)	Vacuum (mm Hg Pressure)	Screen Level (cm from SyringeFilter)	Blue Spots per Plate (4 Leaves)
552×10^3	445	16	1.7 (± 0.3)
552×10^3	597	16	91.7 (± 8.5)
414×10^3	445	16	1.0 (± 0.6)
414×10^3	597	16	70.3 (± 6.9)
414×10^3	445	17.5	4.0 (± 0.6)
414×10^3	597	17.5	51.0 (± 19.9)

Table 2: Analysis of variance for protocol comparison. Data were analyzed as a completely randomized design with a 2 x 2 x 2 factorial arrangement of treatments where two treatments were missing. SC: screen level. HEP: helium pressure. VAC: vacuum pressure.

Source	DF	Mean Square	F-Value
SC	1	200.08	0.74
HEP	1	363.00	1.35
VAC	1	19646.30	72.99*
SC*VAC	1	374.08	1.39
HEP*VAC	1	320.33	1.19
Error	12	269.17	
Corrected Total	17		

*Significant at α=0.05.

Table 3: Comparison of DNA annealing protocols. Tungsten microprojectiles (1 μm diameter) were coated with pAHC25 plasmid DNA by annealing with 40 μl 2.5 M $CaCl_2$ and 20 μl 5 M spermidine or 50 μl 1 M $Ca(NO_3)_2$. For each treatment there were five replications of two leaf halves each. Each replication was bombarded twice to increase GUS expression. Twenty-four hours after bombardment the leaves were incubated in x-gluc overnight (16 h to 20 h) and the GUS spots were counted. Data are presented as the average of five replications.

Treatment	Total Spots	Average No. of Spots Per Leaf
$CaCl_2$ plus Spermidine	303	30.3 (± 12.3)
$Ca(NO_3)_2$	206	20.6 (± 9.1)

Table 4: Analysis of variance of DNA annealing protocol data.

Source	DF	Mean Square	F-Value
Treatment	1	940.90	1.13
Error	8	834.75	
Corrected Total	9		

Osmotic Treatments

Transient GUS expression after microprojectile bombardment was observed on embryos plated on control medium and all media containing osmotic treatments 0.1 M to 0.7 M each of mannitol and sorbitol (Table 5). No GUS expression was observed with the 0.8 M, 0.9 M and 1.0 M treatments. The 0.3 M treatment of each osmoticum produced the highest number of embryos expressing GUS, the highest total number of blue spots, and the highest number of spots per embryo.

A second experiment was conducted to determine if the high expression of the 0.3 M treatment was significantly different from the non-osmoticum treatment. The experiment consisted of bombarding embryos on either the control medium or the 0.3 M osmotic medium and counting the number of GUS spots (Table 6). The results were compared with an ANOVA (Table 7) and there was a statistically significant difference ($P<0.05$) between the two treatments. Only one or two GUS spots were obtained on a few embryos cultured on the control medium (Figs. 3a and 3b). The osmotically treated embryos, however, showed large, dark stained areas (Figs. 3c and 3d) comprised of multiple GUS spots. In addition to the amount of GUS expressed, the 0.3 M osmotic treatment increased the number of embryos expressing GUS.

Table 5: First Osmoticum Experiment. Approximately 30 embryos of various ages were plated on SH-30 medium supplemented with equimolar amounts of mannitol and sorbitol ranging from 0.0 M to 1.0 M . The embryos were cultured on this medium for 24 h before and 24 h after bombardment. They were then incubated in x-gluc and the GUS spots were counted.

Osmotic Treatment (M)	No. of Embryos	No. of Embryos with GUS Spots	Total No. of GUS Spots	Average No. of Spots per Embryo
0.0	27	5	8	0.30
0.1	28	20	55	0.51
0.2	30	20	74	2.47
0.3	33	33	207	6.27
0.4	31	27	131	4.23
0.5	33	15	55	1.67
0.6	30	12	38	1.27
0.7	31	6	22	0.71
0.8	31	0	0	0.00
0.9	28	0	0	0.00
1.0	26	0	0	0.00

Table 6: Second osmoticum experiment. Four replications of thirty embryos at various ages were plated on medium containing either 0.0 M or 0.3 M each of mannitol and sorbitol. The embryos remained on the medium for 24 h before and 24 h after bombardment and each plate was bombarded twice. After treatment the embryos were incubated in x-gluc and the number of GUS spots were counted.

Osmoticum Treatment	Average Number of Embryos with GUS Expression	Average Number of GUS Spots
0.0 M	5 (± 1)	8 (± 2)
0.3 M	23 (± 3)	80 (± 18)

Table 7: Analysis of variance for second osmoticum treatment. Treatments arranged in a CRD.

Source	DF	Mean Square	F-Value
Treatment	1	10585.13	16.29*
Error	6	649.63	
Corrected Total	7		

*Significant at α=0.05.

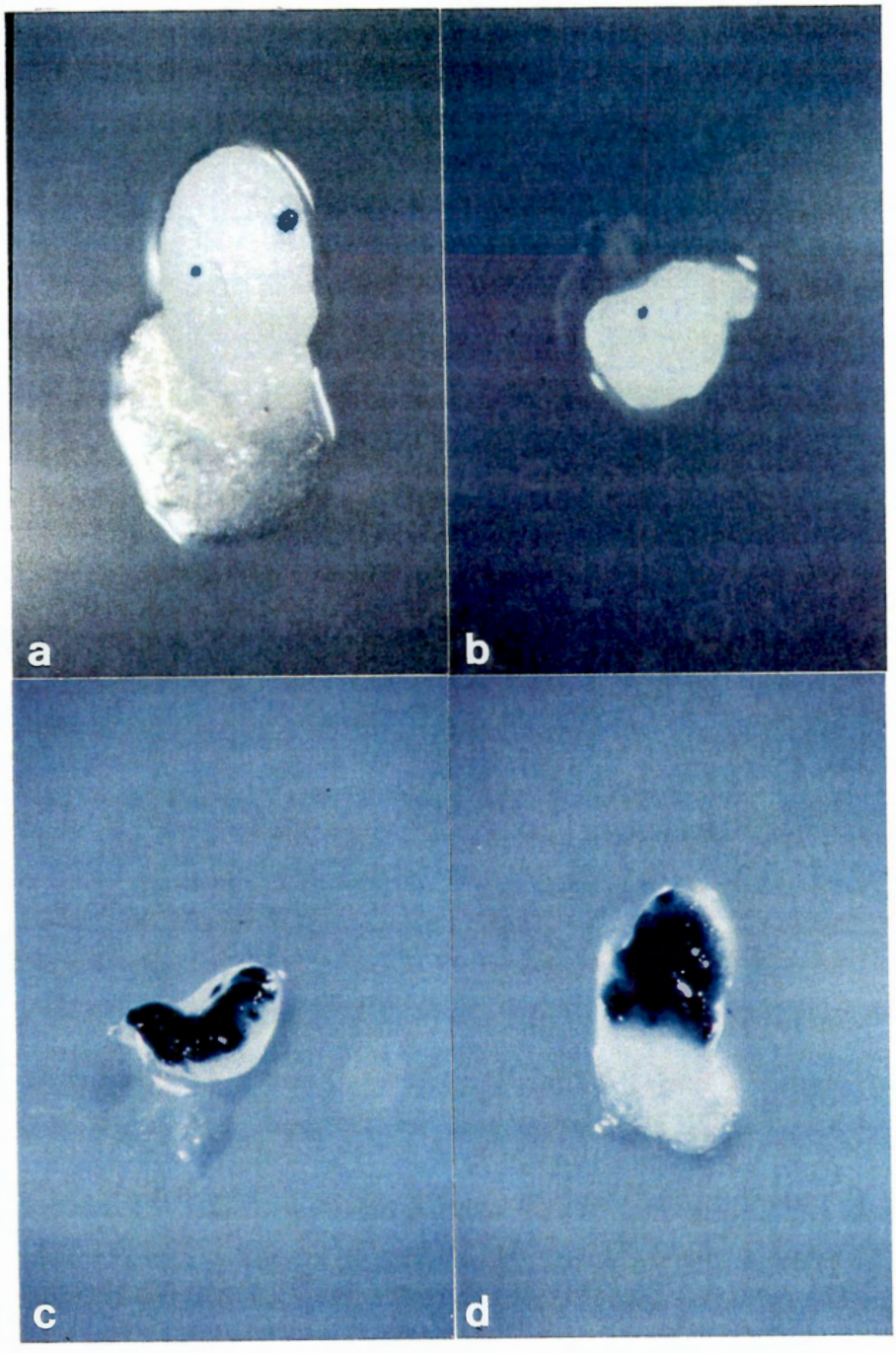


Figure 3: GUS expression in post-bombarded somatic embryos. GUS expression in embryos bombarded on SH-30 medium without osmoticum is shown in (a) and (b). (c) and (d) demonstrate GUS expression in embryos bombarded on SH-30 medium amended with 0.3 M each of mannitol and sorbitol. The embryos were on their respective medium for 24 h before and 24 h after bombardment.

Transient GUS Expression in Suspension Cultured Embryos

The number of embryos from each suspension culture that expressed GUS activity was totaled for each 30 d period and a per plate average was calculated. The data (Fig. 4) show that the number of transformed embryos more than doubled between 30 d and 60 d in suspension and did not decrease through 120 d in culture. After this time the numbers began to decline.

Testing for Basta Tolerance and Southern Hybridization Analysis

A solution of 0.1% Basta™ was brushed onto the leaves of 143 regenerated plants which were all obtained from the 30 d and 60 d sievings of various experiments. Of these, six showed complete tolerance to the herbicide application and ten displayed a localized reaction affecting only the exposed area (Fig. 5). All control plants were found to be sensitive to the herbicide and displayed complete necrosis on tested leaves.

DNA from one control plant from each replication and that from the tolerant plants was isolated and analyzed by Southern hybridization. One putative transformant was observed to contain 0.6 kb (600 bp) segments that

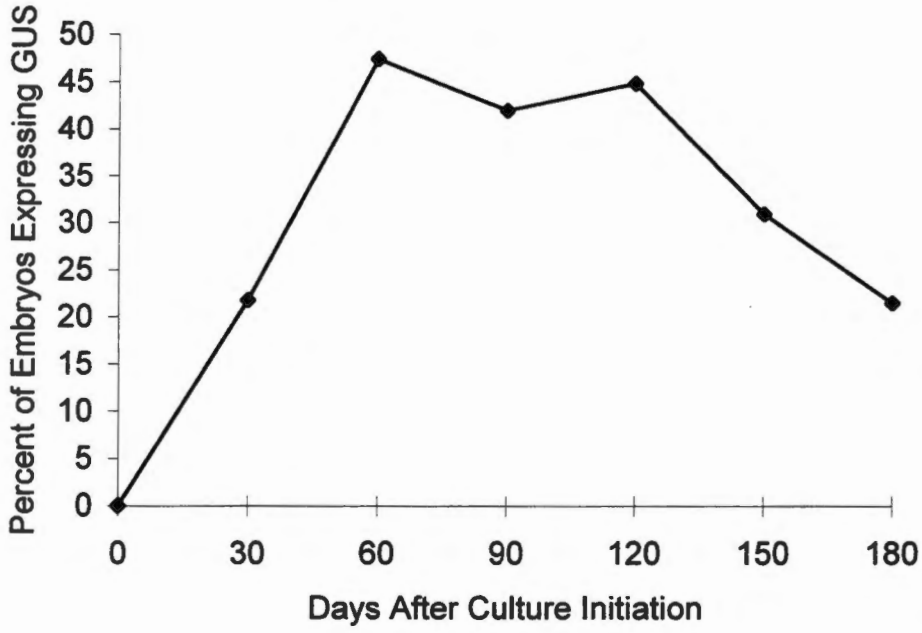


Figure 4: Percent of somatic embryos expressing GUS in suspension culture 0 d to 180 d after culture initiation.

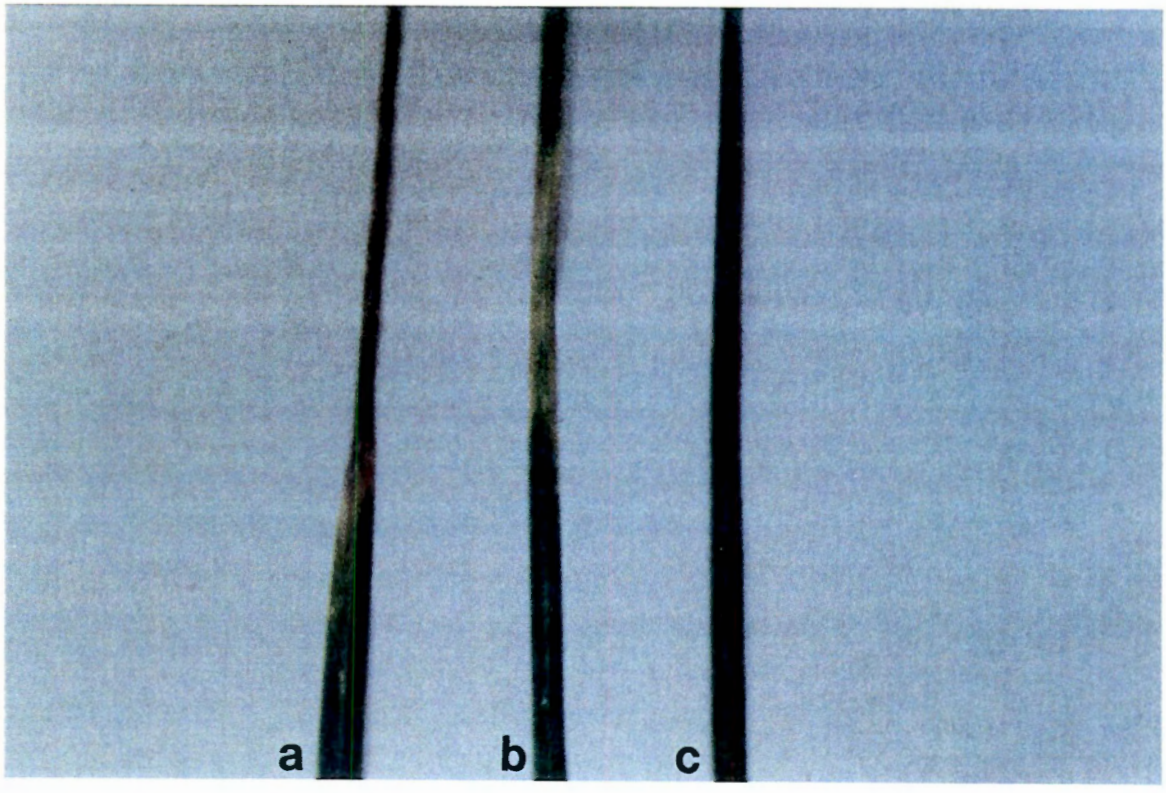


Figure 5: Basta test on leaves of regenerated plants. A solution of 0.1% Basta was brushed on the leaves of 143 plants regenerated from post bombardment embryogenic suspension cultures. A leaf of a nontransformed plant is shown in (a), displaying complete necrosis. (b) Localized reaction on a leaf. (c) Transformed plant showing complete tolerance to the herbicide.

hybridized with the *bar* probe (Fig. 6), thus confirming the presence of the *bar* gene in plant's genome.

Selection Pressure within Suspension Cultures

In fresh weight measurements (Fig. 7), cultures initiated from fractions consisting of smaller tissue mass (two and three) showed a decline in weight over time in all treatments. Cultures initiated from fraction one, however, varied in response according to the medium composition. Fresh weight of the bialaphos treated culture (Fig. 7a) steadily decreased while that of the proline and serine culture (Fig. 7c) increased over the 42 d period. Fresh weight of the casein hydrolysate culture (Fig. 7b) decreased and then increased. Cell viability (Fig. 8) showed an inconsistent pattern of increasing and decreasing over time in each culture (Fig. 9).

Due to a power outage, the experiment was lost 49 d after initiation. The nonviable embryos were salvaged and incubated in x-gluc (see Materials and Methods). Blue spots were counted to determine the number of transformed embryos present in each culture (Table 8). In order to determine the percentage of embryos expressing GUS, the counts were based on 100.

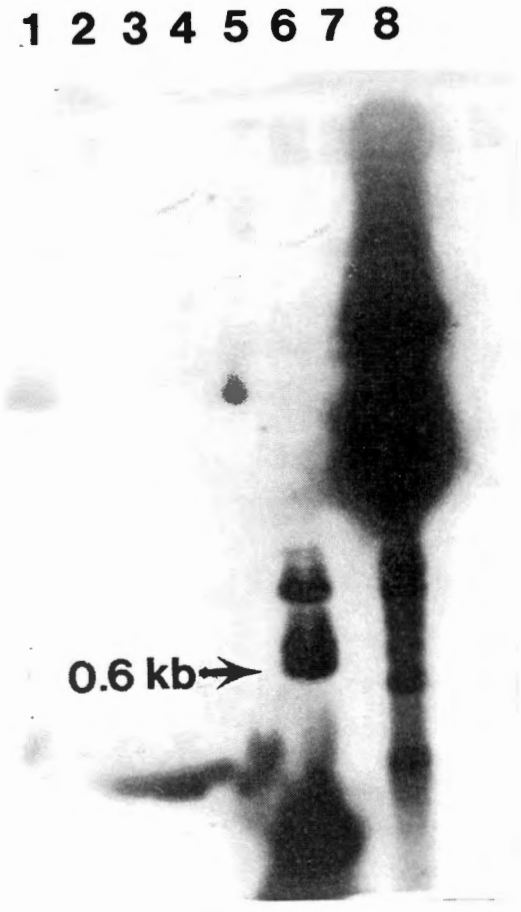
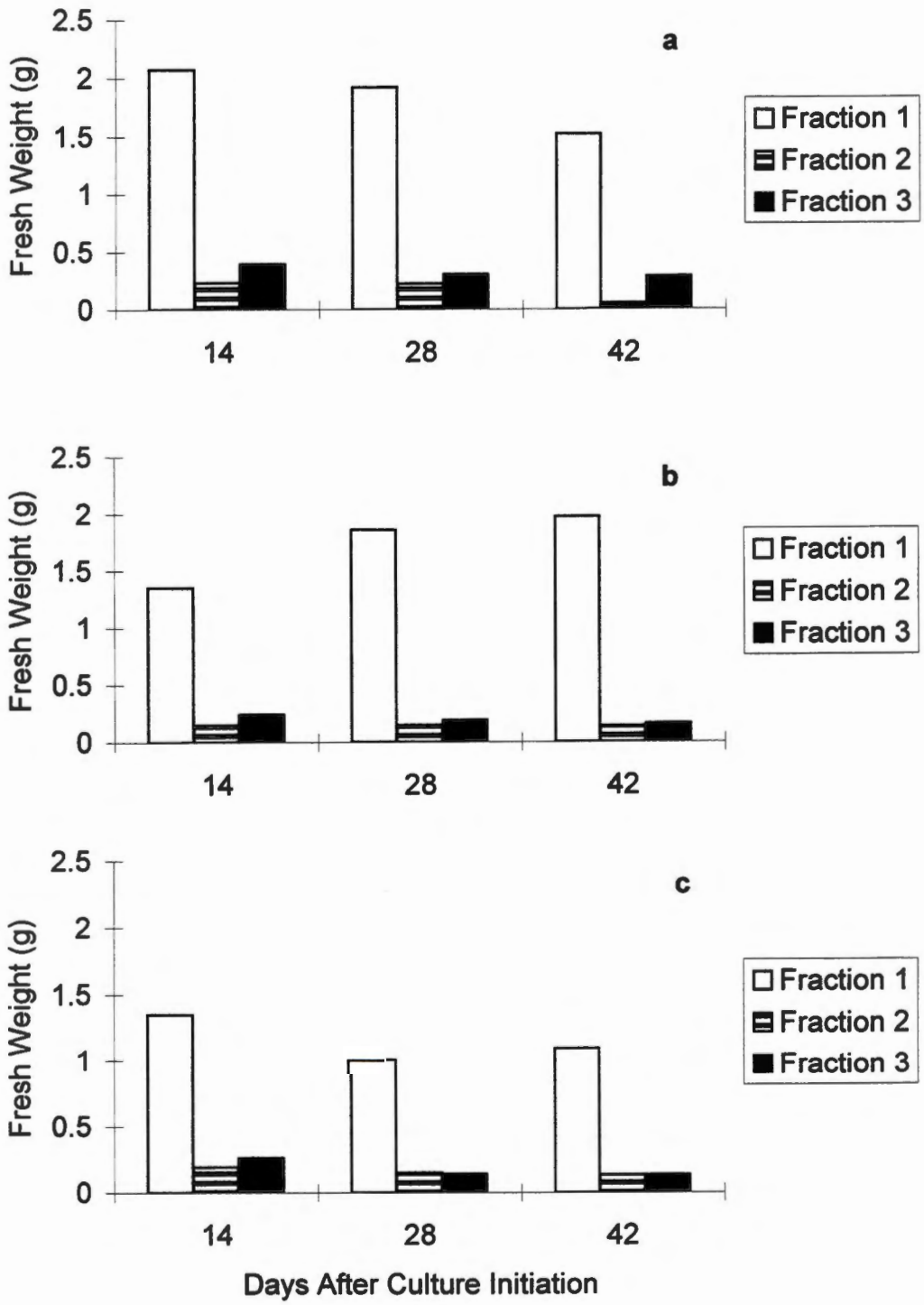


Figure 6: Southern blot analysis of genomic DNA from putative transformed plants. Lane 1: Lambda DNA standard. Lanes 2-6: putative transformants. Lane 7: control plant. Lane 8: pAHC25 plasmid DNA. Lambda DNA was digested with the *Hind* III restriction enzyme. All other digests were accomplished with the *pst* 1 restriction enzyme. The arrow indicates 0.6 kb (600 bp) segments of transformant DNA hybridizing with the *bar* probe and corresponding with the location of the *bar* gene segment in the pAHC25 plasmid.

Figure 7: Average fresh weight (g) of suspension cultures in medium containing (a) bialaphos plus proline and serine, (b) proline and serine and (c) casein hydrolysate. Each culture was sampled at 14 d intervals. This was done by centrifuging the cultures in 50 ml Oak Ridge tubes and removing the medium before measuring the fresh weight. Each graph presents data from cultures initiated from all three cell fractions.


a
b
c
Fresh Weight (g)
2.5
2
1.5
1
0.5
0
14
28
42
Fraction 1
Fraction 2
Fraction 3
Days After Culture Initiation

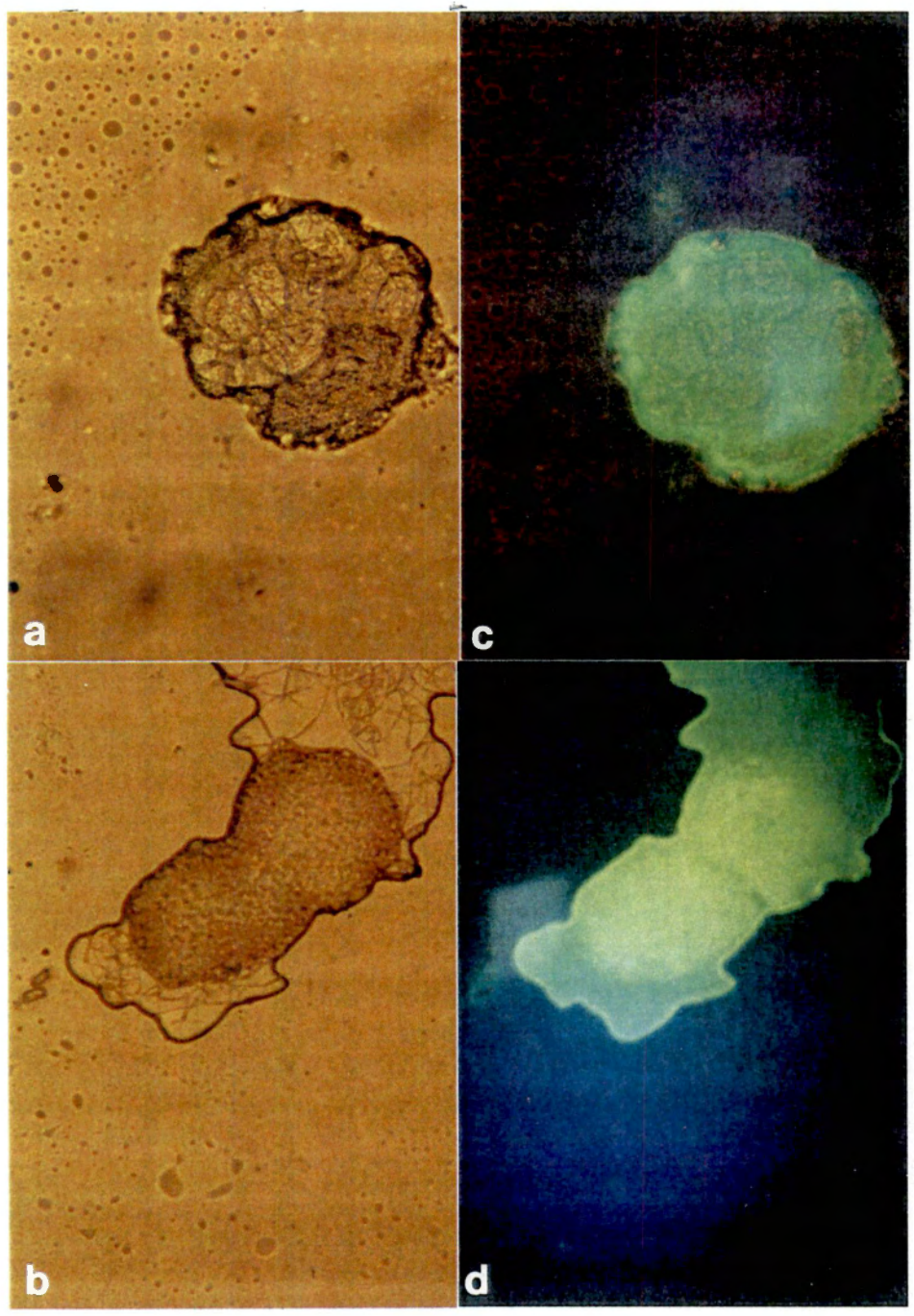


Figure 8: Cell viability tests from putative transformed suspension cultures initiated from fraction one. (a) and (b) show cells and cell clusters from suspension cultures. (c) and (d) show the same cells and cell clusters stained with diacetylfluorescein. The green fluorescence indicates cell viability.

Figure 9: Average number of viable cells per ml in suspension cultures in medium containing (a) proline, serine and bialaphos (b) proline and serine and (c) casein hydrolysate. One ml samples were taken from each of the six replications per treatment at 14 d intervals. The samples were stained with diacetylfluorescein which causes a green fluorescence in viable cells. One thousand cells were counted to determine the viability of the cultures. Each graph presents data from cultures initiated from all three culture fractions.

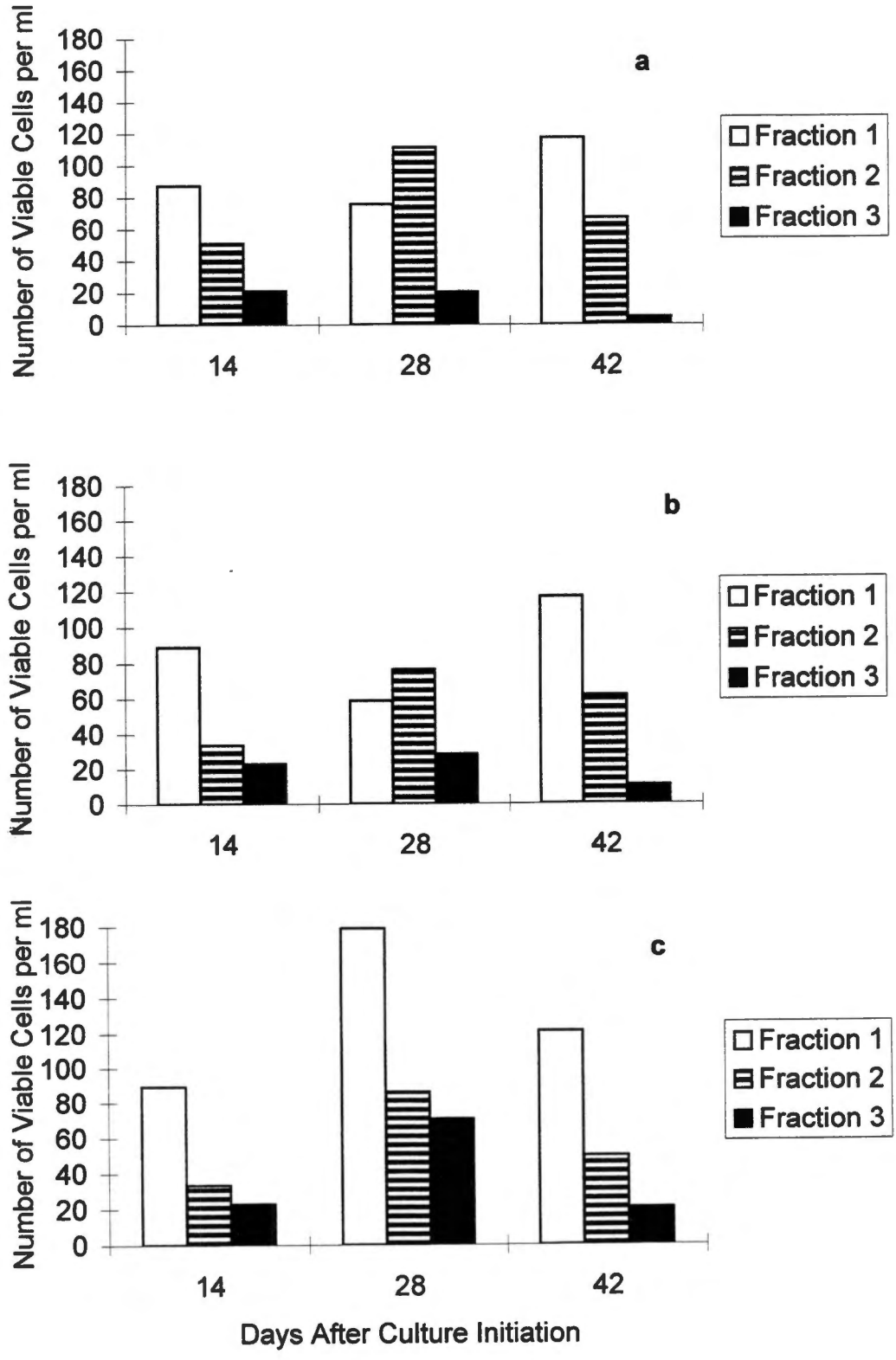

a
b
c
Number of Viable Cells per ml
180
160
140
120
100
80
60
40
20
0
14
28
42
Fraction 1
Fraction 2
Fraction 3
Days After Culture Initiation

Table 8: Average number of embryos expressing and not expressing GUS in 15 ml cultures initiated from different culture fractions. One hundred embryos were counted per culture where possible. B: bialaphos plus proline and serine. PS: proline and serine. CH: casein hydrolysate.

Treatment	Fraction Culture	No. Embryos Expressing GUS	No. Embryos not Expressing GUS
B	1	11 (± 6)	89 (± 6)
B	2	11 (± 5)	75 (± 15)
B	3	2 (± 1)	32 (± 22)
PS	1	31 (± 17)	69 (± 17)
PS	2	22 (± 7)	50 (± 19)
PS	3	30 (± 13)	32 (± 14)
CH	1	61 (± 22)	39 (± 22)
CH	2	3 (± 2)	48 (± 16)
CH	3	1 (± 1)	11 (± 3)

Some cultures, however, failed to produce sufficient embryos for this purpose.

Cultures initiated from fraction one of the casein hydrolysate culture contained the highest number of embryos expressing GUS. Fraction one cultures of casein hydrolysate and all three fraction cultures of proline and serine contained higher numbers of embryos expressing GUS than the bialaphos selection medium. The embryos under selection pressure, however, showed expression which was more uniform throughout the tissue than those in the control cultures.

Chapter Five

Discussion

Results of experiments performed in this study demonstrated that foreign DNA was successfully transferred to somatic embryos of orchardgrass by microprojectile bombardment. Data also showed that transformed embryos placed into suspension culture produced new embryos that expressed the inserted genes.

There were two bombardment parameters that produced a significant increase in transient GUS expression. These were: (1) a vacuum pressure of 597 mm Hg compared to 445 mm Hg and (2) addition of an osmoticum treatment to the pre and post-bombardment culture medium. Many researchers agree that vacuum pressure is an important factor for successful DNA transfer by microprojectile bombardment (Hunold et al., 1994; Morrish et al., 1993; Vain et al., 1993a). Klein et al (1988) observed a 350 fold increase in DNA delivery to maize suspension cells when the vacuum was increased from 10 in Hg pressure to 28 in Hg pressure. They hypothesized that the increase was due to a reduction in air resistance. This allowed the microprojectiles to penetrate the cells more efficiently because of increased velocity as they traveled through the chamber.

In the current study, the addition of 0.3 M each of mannitol and sorbitol to the medium resulted in a more than 10 fold increase in transient GUS expression and also produced a higher number of embryos expressing the

gene. Supplementing the medium with an osmoticum such as sucrose, mannitol or sorbitol as a pre and post-bombardment treatment was also shown by other investigators to produce an increase in transgene expression (Perl et al., 1992; Vain et al., 1993a; 1993b; Hunold et al., 1994; Kuai and Morris, 1995; Semeria et al., 1995; Spangenberg et al., 1995c). Scutellar wheat calli, when cultured both before and after bombardment on medium containing 0.25 M mannitol produced a several fold increase in GUS expression (Perl et al., 1992). In experiments with maize suspension cultures, Vain et al. (1993b) found that by adding mannitol and sorbitol to the medium both before and after tissue bombardment they could obtain up to a 6.8 fold increase in stable maize transformants for bialaphos and glufosinate tolerance. An osmotic pretreatment of short duration (10 to 60 min) and high concentration produced significantly increased levels of transient GUS expression in embryogenic suspension cultures of perennial ryegrass (Spangenberg et al., 1995c).

In the present study, bombardment of embryos resulted in individual transformed cells and groups of cells. These embryos were placed into suspension culture according to a procedure similar to Bomminenni et al. (1993; 1994) in experiments with black spruce and white spruce. A more than two fold increase of embryos expressing GUS was observed between 30 d and 60 d after culture initiation and production remained high up to 120 d. The decline in numbers after this period is consistent with results obtained

with both black and white spruce (Bommineni et al., 1993; 1994). This suggests that there is an optimum time period during which transformed embryos can be produced in and retrieved from suspension cultures. Bommineni et al. (1994) suggest that the decrease observed in older cultures may be due to the rapid cell divisions occurring in liquid culture which in turn results in the loss of any DNA that has not been stably integrated.

There are three common methods to test putative transformants for herbicide tolerance (in this case Basta™). These methods are: (1) to introduce a form of the herbicide as selection pressure in liquid culture, such as used frequently with selection for antibiotic resistance (Finer and McMullen, 1991; Wilde et al., 1992; Spangenberg et al., 1995b;c), (2) add the herbicide to solid medium to screen embryos at the germination stage (DeBlock et al., 1987; Gordon-Kamm et al., 1990; Vain et al., 1993b; Zhong et al., 1993; Hartman et al., 1994; Denchev et al., 1995; Wan et al., 1995; Conger et al., 1996), and (3) apply the herbicide directly to the leaves of putative transformed plants (DeBlock et al., 1987; Fromm et al., 1990; Gordon-Kamm et al., 1990; Cao et al., 1992; Casas et al., 1993; Weeks et al., 1993; Denchev et al., 1995; Vasil, 1992).

Selection on solid medium was attempted, but no embryo germination was obtained. This is presumed to be due to the presence of possible chimeric transformations similar to the transient-GUS spots. Therefore, testing for stable transformation was accomplished by direct herbicide

application to regenerated plants. DeBlock et al. (1987) used this method to test regenerated transformed tobacco. They sprayed plants in the greenhouse with 8 L/ha and 20 L/ha doses of Basta™ and all 21 plants were found to be herbicide tolerant. The same results were reported with duplicate applications of Herbiace™. In experiments with wheat, Vasil et al. (1992) brushed both control and putative transformed plants with 0.001, 0.01, 0.1 and 1.0% solutions of Basta. All control plants browned and died while putative transformants remained green and living. A similar test was conducted by Denchev et al. (1995) by applying a 0.01% solution of Basta™ to the leaves of regenerated transformed orchardgrass plants. Six of 35 putative transformed plants displayed tolerance to the herbicide application. Weeks et al. (1993) tested the first seed produced generation of plants from transgenic wheat for genetic inheritance of Basta tolerance. Of 49 plants tested with an application of 2% Basta, 42 were tolerant to the application.

In the current study, 143 regenerants were tested and one stable transformant was obtained. Presence of the *bar* gene in this plant was confirmed by Southern blot hybridization. The direct application of Basta™ also allowed the detection of a number of plants which exhibited only localized reaction and those which showed herbicide tolerance. However, DNA from these plants failed to hybridize with the *bar* probe. This may be due to chimeric gene expression or may indicate possible genetic escapes (plants which display the transgenic traits without being transformed) in the

culture. It may also be possible that a defense mechanism similar to hypersensitivity has been activated in these plants. This has been shown for resistance to pathogens when plant cells surrounding a pathogen die thereby limiting transport through the tissue (Fritig et al., 1987).

In order to eliminate chimeric expression in embryos, liquid selection experiments were attempted in medium containing bialaphos with proline and serine as the nitrogen source. Similar experiments were conducted with suspension cultures of soybean (Finer and McMullen, 1991) and perennial ryegrass (Spangenberg et al., 1995c) using the antibiotic hygromycin as a selective agent. These experiments are discussed in the literature review.

Fresh weight samples were taken in the current study to determine growth and differences between cultures initiated from different tissue and cell fractions. It was expected that fresh weight would increase over time. Many cultures, however, showed a decrease in weight during the course of the experiment. Slight decreases in weight may be explained by the sampling of cultures every 14 d. This removal of cells, combined with a possible lag in culture growth due to frequent manipulation, may have reduced cell mass.

Decreasing fresh weight may also have been due to the fragmentation of dead cells and their subsequent release into the culture. These dead cells and fragments would have less mass than living cells and may not have

sedimented after centrifugation. Therefore, dead cells and tissue may have been removed with the medium before fresh weights were measured.

All fraction cultures in medium with bialaphos decreased in mass. This can be attributed to the action of the herbicide killing untransformed cells. In the controls, it was observed that tissue mass in cultures initiated from fraction one, consisting of the largest cell masses, increased in both cultures while that in cultures initiated from fractions two and three decreased. This may indicate superior growth in cultures initiated from fraction one, or it may suggest that the larger cell masses are structurally more stable and that cells are less likely to become dislodged after death.

The presence of viable cells in all fraction initiated cultures either increased or decreased in what appeared to be approximate 14 d cycles. This may be the culture growth cycle. It was observed that cultures initiated from fraction one generally had higher viability rates than those initiated from fractions two and three. This effect is likely due to the presence of more cell masses initially in fraction one than in fractions two and three. Most viable cells were observed within such cell masses.

Although the cells in bialaphos culture remained viable throughout, cultures of this treatment contained the fewest embryos expressing GUS. A possible explanation is that, at the time of sampling, the nontransformed cells had begun to die, but the transformed cells had not yet had time to proliferate. In addition, although the number of embryos expressing GUS

was lower than in control media, the expression in bialaphos medium was generally more uniform throughout the tissue. Expression in the control media was predominantly in the form of spots or stained sectors of the embryos. These staining patterns suggest that the bialaphos in the treatment cultures had killed the nontransformed cells within the culture so that only transformed cells were able to develop into embryos.

Chapter Six

Conclusions

Expression of both the *uid*A and *bar* genes in suspension cultured somatic embryos of orchardgrass showed that DNA was successfully transfered to these tissues. Furthermore, Southern hybridization analysis showed that stable integration of the foreign genes was accomplished.

Transformed embryos were obtained over a period 180 d from suspension cultures initiated from putative transformed tissues. Since mature embryos were removed at each sieving, it is concluded that embryos after each 30 d period were the result of new embryo formation. In addition, although there was a decline in production of transformed embryos between 120 and 180 d, GUS activity was observed over the entire culture period.

Furthermore, when bialaphos was introduced into suspension cultures of putative transformed embryos, a visible difference was observed between cultures with and without added herbicide. Although the control cultures (those without herbicide) produced more embryos expressing GUS, the cultures undergoing selection pressure produced embryos that expressed GUS uniformly throughout the tissue.

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