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PROTOPLAST CULTURE AND *AGROBACTERIUM*-MEDIATED TRANSFORMATION OF *ASPARAGUS OFFICINALIS* L. USING SOMATIC EMBRYOGENIC CULTURES

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PROTOPLAST CULTURE AND *AGROBACTERIUM*-MEDIATED TRANSFORMATION OF *ASPARAGUS OFFICINALIS* L. USING SOMATIC EMBRYOGENIC CELL CULTURES

By

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ABSTRACT

PROTOPLAST CULTURE AND *AGROBACTERIUM*-MEDIATED TRANSFORMATION OF *ASPARAGUS OFFICINALIS* L. USING SOMATIC EMBRYOGENIC CELL CULTURES

By

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Plant improvement via biotechnology depends on the ability to regenerate plants from target cells. Protocols based on somatic embryogenesis are ideal for this purpose because somatic embryos originate from single cells and upon germination, a root and shoot are produced from the same structure expediting plant formation. This study examines using embryogenic suspension cultures to expedite plant regeneration from *Asparagus officinalis* L. protoplasts for potential direct-gene-transfer studies, and determines optimal parameters for transient and stable transformation of asparagus embryogenic suspension cells with *Agrobacterium*.

The ability of asparagus protoplasts derived from embryogenic suspension cells to undergo somatic embryogenesis and germinate into normal plants was influenced by genotype and auxin source. Embryogenic suspensions were initiated from four asparagus genotypes and cultured in either 5 µM 2,4-D or 50 µM NAA. There was a significant interaction between genotype, suspension auxin, and inclusion or exclusion of PGR's in the protoplast culture media on plating efficiency and somatic embryo formation. Plating efficiencies at 14 days

ranged from 0 - 40% and globular embryos developed from protoplasts in some treatments in the same amount of time. All four genotypes regenerated plants although Rutgers 22 had the highest germination frequency at 42%.

In an effort to increase the *Agrobacterium* transformation frequency in asparagus, embryogenic suspension cells were targeted and relevant transformation parameters were systematically optimized via transient GUS expression with an intron-containing GUS gene. Embryogenic cells inoculated at 5 x 10^7 cfu/ml with either *A. tumefaciens* strain EHA105 or GV3101(pMP90) that had been induced with acetosyringone and cocultivated for four days was determine to be optimal for transient GUS expression. Selective agents were compared and the cells were most sensitive to glufosinate followed by G418 and kanamycin, respectively. One transgenic plant was produced when the optimal parameters were applied to stable expression using the BAR gene. Transient GUS expression was low when compared to stable expression. This indicated that the T-DNA was entering the cells and being expressed but not integrated into genomic DNA.

DEDICATION

This dissertation is dedicated to my wife Lori. If it was not for her love, support and motivation, this document may have never been completed.

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Chapter One

Literature Review

General Introduction

Origin of Asparagus

The genus *Asparagus* L. belongs to the Liliaceae (lily) family. There are between 100 and 300 species of perennial herbs, woody vines or shrubs in the genus distributed throughout the temperate and tropical regions of the world (Bailey and Bailey 1976). The name asparagus probably originated from the old Iranian word "sparaga" which means shoot, rod or spray. The Greeks used the word "aspharagos" which means to "sprout" or "shoot". This was converted to the common name "asparagus" which has been used by the Romans and other European nations (Luzny 1979). *Asparagus officinalis* L. is the only species of asparagus that has been domesticated for edible consumption and is considered to be an important commercial vegetable worldwide. It is a European-Sibirican continental plant related to the East Mediterranean vegetation (Reuther 1984). As a commercial crop it is native to the Orient and to the eastern parts of the Mediterranean center of crop origin. The Greeks introduced it into their homeland from the eastern nations and subsequently the Romans adapted its culture from the Greeks (Luzny 1979). During the Imperial Rome era, it was a popular vegetable. It was probably the Roman Legions that subsequently introduced it to central Europe. This assumption is based on the fact that wild asparagus is now localized in the vicinity where the Roman Legion camps were situated (Luzny 1979). Indigenous asparagus

species are described in different regions in Europe. Henderson (1890) described it as native to Great Britain, Russia and Poland. It is suggested that the native forms of asparagus originated from cultivated types which then reverted to wild forms (Luzny 1979). In Great Britain, asparagus grew wild in most vegetable gardens (Sturtevent 1919). From there, asparagus arrived in New England with the Puritans in the early seventeenth century. In the U.S. it escaped cultivation and became adapted to sandy fields, road sides and garden sites. By 1776, asparagus was growing in every colony along the Atlantic coast. During the following century it became widely distributed in North America. Today, asparagus is grown commercially on every continent except Antarctica. The United States is the leading producer of asparagus with production being led by California and followed by Washington and Michigan (Nichols 1990; Desjardins 1992).

Botanical description

Asparagus officinalis is a long-lived monocotyledonous, herbaceous perennial, and it is dioecious. The plant is grown for its succulent fleshy shoots (spears), which appear after a prolonged winter rest period. The crown of the plant is the growth center from which the spears arise. The plant consists of underground stems (rhizomes), fleshy roots, and fibrous roots. The fleshy roots serve as storage organs and the fibrous roots as absorption organs. As each growing season

progresses, the rhizomes develop new buds that generate the spears of the following year. The spears consist of short internodes and lateral buds covered with bracts. The bracts are the true leaves but do not have photosynthetic capability. Instead, photosynthesis occurs in the cladophylls (Peirce 1987). The cladophylls are the fern-like structures that develop from the lateral buds. Structurally, a cross section of a spear shows five anatomical regions: the epidermis, cortex, pericyclic fibers, ground parenchyma, and vascular bundles (Peirce 1987). From the center of the asparagus plant there is a progressive lateral growth of rhizomes and roots of several centimeters per year. This growth can produce a rhizome radius of 60 cm or more in 10 - 15 year-old crowns (Nonnecke 1989).

Asparagus is dioecious with 2n = 2x = 20, and produces male and female florets on separate staminate and pistillate plants. Sex determination is controlled by a single locus (Franken 1970) located on the L_5 chromosome (Loptien 1979). Female plants are homogametic (mm) and males are heterogametic (Mm). Upon crossing, the ratio of pistillate to staminate plants is 1:1. Female flowers from different plants are similar in pistil and stamen ontogeny whereas, male flowers can vary greatly in pistil development (Franken 1970; Galli et al. 1993). Pistil development in male flowers can range from rudimentary to fully functional. Male flowers with functional pistils are termed hermaphroditic and the male plants that

bare them are andromonoecious (Franken 1970). Upon self-pollination, a hermaphroditic flower will give rise to a progeny ratio of 1:2:1 homogametic supermale (MM), heterogametic male (Mm), and homogametic female (mm), respectively. Supermales are very important in asparagus breeding programs because the result of crossing a supermale and a female is an all male population. Generally, male plants give rise to more spears and are consequently higher yielding than female plants (Franken 1970). It should be noted that supermales can also be produced via anther culture (Falavigna et al. 1996).

Breeding Goals

Asparagus has an average harvest period of 10 years and also demands a great amount of financial and labor input before any economic return is realized. Therefore, improvement of cultivars by breeding has high priority. Important breeding aims are all-male hybrids, high yield and quality characterized by an increased number of spears of large diameter per plant, uniformity among the spears, low level of fiber in the spears, high disease resistance, salt tolerance and climatic adaptability (Reuther 1984). In the Netherlands, breeding for earliness is important because of the cool climate. Earlier cultivars would allow the harvest to occur along with Southern Europe which is typically a month sooner (Scholten and Boonen 1996).

Fusarium is considered the most limiting factor in asparagus production (Mace et al. 1981; Peirce 1987). *Fusarium oxysporium* (Schlecht) f. Sp. *asparagi* Cohen (FOA) is the causal agent of the wilt and root rot disease and *Fusarium moniliforme* Sheld. emend. Snyd. & Hans (FM) is the causal agent of stem and crown rot (Cohen and Heald 1941; Johnston et al. 1979). Because of fusarium, asparagus plantings decline to uneconomical levels as soon as 5-6 years due to the reduction in plant vigor and loss of crowns. Replanting in fields where asparagus was grown previously results in losses of up to 50% of the new plants within the first year. This problem is described as the "asparagus decline and replant syndrome" (Hanna 1947; Lake et al. 1993). Because FOA and FM are transmitted through soil, the use of fungicides and fumigation for long-term control is limited. So, breeding for disease resistant varieties is the most effective way for control of this disease. Takatori and Southern (1978) in California and Ellison (1986) in New Jersey made attempts to select for asparagus resistance to fusarium, but to date no such cultivar has been developed. However, new lines introduced from Rutgers such as Jersey Giant have considerable tolerance to this disease. Ellison (1986) attempted to use exotic germplasm in breeding for fusarium resistance. He collected numerous seed samples of *A. acutifolius* in the wild from Greece, Italy and Spain and seed of wild *A. maritimus* from Yugoslavia. Unfortunately, all of those accessions were

found to be susceptible to fusarium. Only ornamental species such as *A. densiflorus* cv. Sprengerii and *A. plumosus* have been found to be highly resistant to fusarium (Lewis and Shoemaker 1964; Stephens et al. 1989), but neither of them hybridize sexually with *A. officinalis* (Ellison 1986).

In addition to fusarium, rust (*Puccinia asparagi*), phytophthora spear rot (*Phytophthora* spp.), botrytis blight (*Botrytis cinerea*), stemphylium leaf spot (*Stemphylium vesicarium*) and asparagus virus 1 (AV1) and asparagus virus 2 (AV2) effect asparagus production. These diseases are not as ubiquitous as fusarium but can severely affect production if conditions permit (Peirce 1987). With the exceptions of fusarium and viruses, the fungal diseases can be controlled by using resistant varieties, fungicides and cultural practices (Broadhurst 1996; Scholten and Boonen 1996).

There are several differences in the growth of female and male plants that make the males more desirable for field production. Male plants give rise to more spears and are consequently higher yielding than the females. Female spears generally are heavier individually but fewer in number than in male plants (Ellison and Scheer 1959). Also, male plants do not produce seedlings which compete with the established crowns for nutrition and may favor disease epidemics. For these reasons, breeding efforts are concentrated on developing all-male cultivars that will produce higher, more stable yields of spears (Ellison 1986).

Somatic embryogenesis - An overview

Somatic embryogenesis is the process by which haploid or diploid somatic cells develop into differentiated plants through characteristic embryological stages without fusion of gametes (Williams and Maheswaran 1986). This process occurs *in vivo* but is confined to intra-ovular tissues including the nucellus, inner integument, synergids, antipodals and endosperm (Tisserat et al. 1979). Currently however, somatic embryogenesis is best known as an *in vitro* developmental pathway. *In vitro* somatic embryogenesis was first observed in carrot cell cultures where structures resembling zygotic embryos, "embryoids", developed and gave rise to plants (Reinert 1958; Steward et al. 1958). These structures were eventually termed somatic embryos because they developed from somatic cells without gametic fusion (Ammirato 1987).

Somatic embryos are characterized as structures that progress through morphologically defined stages of development (globular, heart-shaped, torpedo and cotyledonary) and culminate in the formation of a bipolar structure possessing both a shoot and root apex at maturity (Ammirato 1987). Somatic embryos do not have endosperm due to the lack of gametic fusion and are generally genotypically identical to the donor parent if derived from somatic cells. Also, seed coats are not present on somatic embryos since they are the result of the integuments hardening around the zygotic embryo. Somatic embryos can be distinguished from shoots *in vitro* by

the presence of a "closed" vascular system connecting both apices. The vascular system of a shoot or a root extends into the parental tissue. In addition, embryos give rise to cotyledons prior to true leaves while shoots produce only leaves (Haccius 1978).

In vitro somatic embryos develop via either direct or indirect embryogenesis (Sharp et al. 1980; Evans et al. 1981). Direct embryogenesis involves the development of embryos directly from tissues without an intervening callus. The embryos arise from "pre-embryogenic determined cells" (PEDC's) which are embryogenic at the time of explanting and undergo a consecutive mitotic development sequence characteristic of zygotic embryogenesis when environmental conditions are favorable. PEDC's are most prominent in embryonic tissues such as cotyledons, hypocotyls and the embryonic axis, but can also occur in leaves, stems and other non-embryo associated explants. In indirect embryogenesis, cells must dedifferentiate before they can express embryogenesis. Dedifferentiation of non-PEDC's converts them to induced embryogenic determined cells (IEDC's) which are embryogenically competent under the proper conditions. IEDC's are primarily present in embryogenic callus and suspension cells.

Auxin is the most important factor for the regulation of induction and development of embryogenesis and has different effects on different phases of embryogenesis (Komamine et al. 1992). Auxin is required for the formation of embryogenic

cell clusters from non-induced competent cells. Once IEDC's are formed, the same level of auxin required for induction of embryogenesis is inhibitory for expression of embryogenesis. IEDC's will express embryogenesis upon auxin removal.

Asparagus Biotechnology

Somatic embryogenesis

The first report of somatic embryogenesis in a monocot was with *Asparagus officinalis* by Wilmar and Hellendoorn (1968). Totipotency of cultured plant cells had only recently been demonstrated in dicots (Steward *et al.*, 1958) and so asparagus was chosen to determine if monocots also had the ability to develop whole plants from undifferentiated cells. A green callus was induced on hypocotyls from sterile seedlings incubated on LS medium with 1 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.315 mg/l kinetin. Upon omission of the growth regulators, shoots and some roots developed from the calluses similar to previous reports with carrot (Steward et al. 1958). To investigate embryo development in more detail, callus cells were placed in LS liquid medium containing the same levels of 2,4-D and kinetin and agitated on a shaker. Small cytoplasmically dense cells developed along with globular embryos in suspension and these embryos developed further as the 2,4-D concentration was reduced. Steward *et al.* (1958) observed that the embryos developed according to the pattern of zygotic embryos *in vivo*

and many eventually elongated to form banana-shaped structures up to 2 mm in length. They also described polarity, a criteria for embryogenesis, as an increase in chlorophyll content on one side of the embryos and elongation to form the radicle on the other. Some embryos germinated into plants when placed on medium lacking PGR's although transferring the embryos at the right stage from suspension to this medium was critical. Normal diploid plants were obtained as well as some tetraploids.

During the same period, Steward and Mapes (1971) were also investigating totipotency in asparagus and the possibility of developing a mass propagation system for the crop. Asparagus was considered to be recalcitrant since it is a monocot, and a challenge to establish in tissue culture. The authors proposed that if a propagation system from free cells could be developed, elite genotypes could be indefinitely multiplied. Stem segments of asparagus were placed on White's medium with 10% coconut water and NAA to establish callus cultures. Subsequently primary calluses were placed in liquid MS medium with coconut water and 2,4-D to produce morphogenic suspension cultures. NAA satisfactorily promoted callus growth, but produced little evidence of organized growth, whereas 2,4-D resulted in more regular and organized cell clusters. It was believed that 2,4-D provided a morphogenic stimulus necessary for embryogenesis and that NAA did not. When the cells were transferred to liquid medium

containing NAA, "embryoids" and cell clusters with roots developed. If the 2,4-D cultured cells were transferred to MS medium containing only coconut water, vigorous root growth occurred. However, when the cells were transferred to liquid MS medium alone, the cell clusters gave rise to numerous feathery shoots. These feathery shoot cultures could be placed on semi-solid MS medium and rooted. This study had shown that cultured asparagus cells had all the genetic information and all the cytoplasmic machinery to support their totipotent development given the proper stimuli. This report demonstrated that a clonal propagation system via somatic embryogenesis was possible in asparagus.

Levi and Sink (1990; 1991; 1992) investigated several factors influencing the induction, development and subsequent conversion of somatic embryos in *Asparagus officinalis* L. The effects of carbohydrates on embryogenesis were tested (Levi and Sink 1990). Calluses were incubated on induction medium containing LS salts, 1.5 mg/l 2,4-D and 0.3 mg/l 2iP or 1.5 mg/l NAA and 0.3 mg/l N^6-(2-isopenntenyl)adenine (2iP), and 2, 3, 4, 5, or 6% (w/v) of either sucrose, glucose or fructose for four weeks. Callus tissues were subsequently placed on subculture media containing LS salts, 0.08 mg/l NAA, 0.2 mg/l 2iP and the same sugars as above. Upon subculture, calluses were placed on all combinations of the three sugars (nine treatments per level) at the various levels. Glucose induced the greatest number of globular embryos on both 2,4-D

and NAA although the frequency was not significantly different from sucrose on NAA containing medium. Embryo induction generally increased at carbohydrate levels up to 3% and then significantly decreased at concentrations of 5% or higher. Regardless of auxin type, fructose induced the fewest embryos and those embryos tended to be large and vitrified. In general, upon subculture as the carbohydrate concentration increased, so did the development of bipolar embryos. It was finally determined that when 2,4-D was used that sucrose in the induction medium and fructose in the subculture medium gave rise to the most plantlets. When NAA was used, glucose in the induction medium and fructose in the subculture medium was best. The results also showed that more embryos converted into plants when NAA was used in the induction medium compared to 2,4-D.

Levi and Sink (1991) found that the choice of explant and growth regulator can significantly effect embryogenesis and plant regeneration. Embryogenic callus was derived from spear cross sections (SS), *in vitro* crowns (IVC) and lateral buds (LB) on either 0, 0.01, 0.1, 1.0 or 10 mg/l 2,4-D or NAA and 0, 0.1, 1.0 or 10 mg/l kinetin. 2,4-D at 1-10 mg/l in combination with 0-1 mg/l kinetin induced more globular embryos from all three explant sources than NAA at the same concentrations. NAA promoted a higher frequency of bipolar embryo development compared to 2,4-D after the cultures were placed on embryo development medium (EDM). In addition,

bipolar embryos initiated on NAA media converted into significantly more plants than from 2,4-D derived embryos. 2,4-D promoted the development of abnormal embryos especially at 10 mg/l which was noted to be the most likely cause for low embryo conversion. In regards to explant source, SS cultured on 2,4-D yielded the most globular embryos, bipolar embryos and plantlets per gram of callus than IVC and LB explants. When NAA was used to initiate embryogenesis, LB explants produced the greatest number of globular embryos, bipolar embryos and plantlets. The authors concluded that induction of embryogenic callus was best carried out from LB explants on 10 mg/l (50 µM) NAA. This scheme enhanced embryogenesis over other sources and promoted conversion of normal plantlets on embryo development medium. Histology was performed on some of these embryos and their ontogeny was compared to that of asparagus zygotic embryos (Levi and Sink 1991). The authors identified embryogenic cells within asparagus callus that were cytoplasmically dense in contrast to the surrounding cells and tracked their development up to the mature bipolar stage. Mature somatic embryos either closely resembled their zygotic counterparts having a banana or crescent shape with an elongated cotyledon and a lateral shoot apex, or they had a short and wide cotyledon that did not resemble the zygotic embryos. Both types were reported to convert into plantlets with equal frequencies.

In a final study, Levi and Sink (1992) re-examined the production of asparagus somatic embryos from suspension culture and investigated the effect of NAA on embryo induction along with the effect of carbohydrate concentration and type on embryo maturation and conversion. Their attempts to utilize previously published protocols (Wilmar and Hellendoorn 1968; Steward and Mapes 1971) resulted in mostly abnormal structures and few plantlets. These earlier reports had also failed to quantify embryogenic responses nor mention the germination frequency of embryos to plantlets. Callus cells were placed into liquid MS medium with NAA and 2iP at non-embryogenic levels to establish suspension cultures. Cells from these suspensions were placed in liquid MS medium containing 0.54, 5.4, 16, 54, 81 or 107 µM NAA. The suspensions were evaluated for embryogenic potential by assessing (1) the frequency of single elongated vacuolated cells, (2) the number of organized clusters of cells with enriched cytoplasm, (3) the number of proembryogenic masses consisting of elongated vacuolated cells and globular embryos at early developmental stages (8 to 64 cells), and (4) the quantity of translucent globular embryos with a smooth spherical profile. NAA at 16 µM or below produced suspensions that contained mostly single, elongated vacuolated cells which is characteristic of a lack of embryogenic potential. These suspensions also contained some clusters of cytoplasmically dense cells and many abnormal embryos that failed to convert

into plantlets on embryo development medium. In contrast, suspensions containing NAA at 54 µM or higher were quite embryogenic. They also possessed some elongated cells along with many clusters of dense cells with organized divisions and globular embryos. Although less than 50% of the initial suspensions at these levels were embryogenic, those that were gave a high frequency of mature bipolar embryos and vigorous plantlets. Based on these observations, the authors state that levels of NAA at 54 µM or higher are required to induce a high frequency of embryogenesis in asparagus cells and that stringent selection of cell characteristics must be used to obtain and maintain embryogenic suspensions. To evaluate the effect of carbohydrate type and concentration on embryo development, suspension cells were placed on LS medium containing either sucrose at 0.06, 0.12, 0.18, 0.24 or 0.3 M, or glucose or fructose at 0.11, 0.22, 0.33, 0.44 or 0.55 M for two weeks and then subcultured to the same sugar at 0.06 or 0.11 M for an additional four weeks. Low sugar concentrations supported secondary embryogenesis, whereas, higher levels promoted the development of bipolar embryos with an average size that correlated to the increase in carbohydrate molarity. Fructose or sucrose concentration sequences of 0.33 to 0.11 M and 0.24 to 0.06 M, respectively, were optimal for the production of vigorous plantlets after two to four weeks on embryo development medium.

Somatic embryogenesis of asparagus has been viewed as a suitable alternative to clonal propagation via adventitious shoots. Kohmura et al. (1994) established a micropropagation system in which bud clusters derived from shoot apices gave rise to somatic embryos. The bud clusters were established by incubating shoot apices on MS medium supplemented with 10 mg/l ancymidol for several months. The compact structures or bud clusters were placed on 10 µM 2,4-D and formed embryogenic calli after several months with bimonthly subcultures. The callus was placed in liquid LS medium without growth regulators on a rotary shaker to allow for somatic embryo development. Mature embryos germinated into plantlets when placed on solid medium without growth regulators. The bud clusters could be maintained indefinitely on ancymidol which allowed for a constant source of tissue for induction of embryogenic callus. The authors established over 2000 somatic embryo derived plants in the field and did not observe the occurrence of any abnormal plants. Cytology was performed on a random sample of plants and all had the expected $2n = 2x = 20$ chromosomes.

Odake et al. (1993) investigated the use of suspension cultures to micropropagate asparagus via somatic embryogenesis. NAA at 3 mg/l in combination with kinetin at 1 mg/l was used exclusively to initiate and maintain embryogenic cultures. Full strength MS medium was most effective for embryogenesis when compared to half-strength MS

or B5 medium. Embryos were placed onto half strength MS medium where most were reported to germinate normally with shoots and roots. Most plantlets were successfully transferred to the greenhouse and acclimatized. Cytology on 33 plants revealed that over 90% were tetraploid while the original stock plants were diploid. The authors speculated that the growth regulators used may have been responsible for chromosome doubling.

Saito et al.(1991) investigated several factors to increase the quality of asparagus somatic embryos and improve germination frequency. Both NAA and 2,4-D were used separately to induce embryogenic callus from asparagus epicotyls. 2,4-D was used in most of the study because the frequency of embryogenic callus initiation from NAA was very low. The granular yellowish white callus that developed was placed in liquid MS with 5 µM 2,4-D where a fine embryogenic suspension rapidly developed. The suspensions were plated onto solid or liquid MS medium without plant growth regulators (PGR's) to determine if medium condition influenced embryo development. Three times as many elongated bipolar embryos developed on solid medium versus liquid medium. The effect of the vessel capping material and Gelrite concentration on embryo maturation and conversion was investigated. Vessels containing embryogenic cultures on 0.2, 0.5 or 1.0% Gelrite were sealed with aluminum foil or a ventilative membrane. The highest Gelrite concentration was best for either closure,

however the use of a ventilative filter drastically increased the number of elongated embryos and plantlets that formed. The relative humidity inside of the vessels with the ventilative filters was lower that those sealed with foil and Parafilm®. In addition, the moisture content of the embryos from the ventilated vessels was 6% lower than the foil closed containers. The use of Gelrite and the ventilative filter had a desiccating effect that prevented vitrification and proved beneficial for embryo maturation and conversion.

Fourteen genotypes of *A. officinalis* were tested for their ability to exhibit somatic embryogenesis from several explant sources (Delbreil et al. 1994). Shoot apices, cladophylls and isolated mesophyll cells were incubated for one month on 10, 1 or 0.1 mg/l NAA, respectively. Following the induction period, the tissues were subcultured to PGR-free medium for embryo development. All tissue sources from each genotype were able to produce embryogenic callus at a frequency of 1-20%. The embryogenic callus which was composed of mostly globular embryos could be subcultured monthly onto medium lacking PGR's and maintained as long-term embryogenic lines. These lines were considered to be habituated and continuously produced embryos although only some were able to convert into plants from ten of the genotypes. Histological studies showed that the long-term cultures grew by recurrent embryogenesis with each new embryo arising from a single epidermal cell of a pre-existing embryo.

In a related study, the same authors found that some plants regenerated from the long-term cultures exhibited a high embryogenic response when recultured (Delbreil and Jullien 1994). When shoot apices from somatic embryo-derived plants (R0) were placed back on embryo induction medium, the frequency of embryogenic callus formation ranged from 16.5 to 91.3%. In addition, these same R0 lines produced embryogenic callus at a frequency of 26 to 57% when the explants were placed on PGR-free medium. None of the control plants that the R0 plants were derived from produced embryogenic callus on PGR-free medium. To determine if the high embryogenic trait was a stable mutation and sexually transmitted, a high embryogenic R0 line was crossed to a low embryogenic line and the F_1 progeny backcrossed to the low embryogenic line. They found that a stable mutation had occurred at a single locus and observed segregation ratios of 1:1 in the F_1 as well as the BC_1 populations. The authors state that if this high embryogenic trait could be transmitted to desirable genotypes without disrupting existing asparagus agronomic characteristics that a very efficient micropropagation system could be developed.

Somatic embryogenesis has been achieved in *Asparagus cooperi* Baker which has medicinal as well as horticultural value (Ghosh and Sen 1991). Callus was initiated from spear sections on MS medium with 1 mg/l NAA and kinetin. After three monthly subcultures, the callus could be induced to form

embryos when placed on the same medium with 2.9 g/l potassium nitrate. Subsequently, the influence of organic and inorganic nitrogen sources on multiplication of this embryogenic callus was investigated. The addition of 300 mg/l glutamine, 1000 mg/l casein hydrolysate and 1200 mg/l ammonium nitrate to the basal medium produced the greatest enhancement of the callus multiplication rate. Mature embryos failed to germinate when they were transferred to MS medium lacking PGR's. However, transferring embryos to 1 mg/l zeatin promoted embryo conversion with a maximum conversion frequency of 38%. Cytology was performed on 80 plants and all were karyotypically normal and free of any noticeable phenotypic variability. The authors further investigated encapsulating *A. cooperi* somatic embryos in alginate to produce synthetic seeds (Ghosh and Sen, 1994). A conversion frequency of over 30% was achieved from encapsulated embryos by the combination of 3.5% sodium alginate (Sigma) and 50 mM calcium chloride whereas naked embryos converted at 45% under similar conditions. When embryos were stored at 4°C for up to 30 days, the germination frequency was higher for the naked embryos. However, encapsulated embryos stored for 60 and 90 days had a higher conversion frequency.

In addition to studies that attempt to optimize the production and development of asparagus somatic embryos, somatic embryos or embryogenic cultures have themselves been used to optimize other technologies or facilitate the recovery

of genetically unique plants. Cryopreservation systems were developed to store asparagus somatic embryos and embryogenic cultures over long periods of time (Uragami et al. 1989; Nishizawa et al. 1992; 1993). Somatic embryogenesis has been used successfully to recover plants from protoplasts (Kunitake and Mii 1990; Mukhopadhyay and Desjardins 1994a; 1994b; May and Sink 1995), haploid plants from anther and microspore culture (Feng and Wolyn 1991; 1993; 1994), and transgenic plants from *Agrobacterium* inoculated embryogenic calluses (Delbreil et al. 1993).

Protoplast culture

Bui-Dang-Ha and Mackenzie (1973) developed the first asparagus protoplast culture scheme in hopes of using it for mass producing valuable cultivars and for accepting foreign genetic material for crop improvement. Protoplasts were isolated from cladodes (fern tissue) from a single cultivar. In many cases, at least 30% of the initial protoplast population died within the first three days. Those that survived achieved first division by eight days although this was quite variable in that some isolations did not divide until 17 days. They reported that 4% of the populations would at least divide once but actual plating efficiency (PE) was not presented. In an attempt to enhance protoplast division, PGR's were added at various concentrations without success. Sustained divisions were only achieved after glutamine was

increased from 200 mg/l to 1000 mg/l. The authors mentioned that a reduced nitrogen source such as glutamine may be beneficial to growth whereas ammonium as the sole nitrogen source may be inhibitory. Selected colonies were placed on solid MS medium and adventitious shoot regeneration was achieved.

A subsequent paper concentrated on plant regeneration from protoplast-derived calluses (Bui-Dang-Ha et al. 1975). Calluses incubated solely on 2,4-D or NAA at equimolar concentrations exhibited callus proliferation and prolific rooting. The addition of BA to either NAA or IAA initiated shoots while using zeatin or 2,4-D in any combination was ineffective. The combination of IAA and BA produced up to 90% shoot initiation in protoplast-derived calluses whereas up to only 20% shoot initiation occurred on NAA and BA. The authors also observed somatic embryogenesis from the calluses. If the calluses were grown on equimolar BA and NAA with 40 mg/l adenine sulfate for 6 - 8 weeks and then subcultured to medium lacking PGR's, a friable callus formed at the margins of about 15% of the calluses. This new callus was composed of various stages of somatic embryos. The embryos required IAA and zeatin for germination into plants because embryos left on medium lacking PGR's failed to develop further. Histological evidence showed the similarity of the somatic embryos to zygotic asparagus embryos.

Elmer et al.(1989) utilized callus cultures from four asparagus genotypes to investigate protoplast isolation, culture and shoot regeneration parameters. Callus was derived from spears of genotypes Jersey Giant #8, Jersey Giant #14, E2 and A19 on six different media containing various combinations of NAA, 2,4-D, BA and/or kinetin. The greatest protoplast yield came from callus grown on 2.5 mg/l 2,4-D and 1.0 mg/l kinetin, and 20 days following subculture versus 10 or 30 days. Jersey Giant #8 (JG8) was the only genotype whose protoplasts were capable of sustained divisions and colony formation. Protoplasts from the other three genotypes died within 3-4 weeks after plating. Protoplasts were cultured in liquid Kao and Michayluk (1981) medium (KM medium) containing several combinations and concentrations of NAA, 2,4-D and BA. JG8 protoplasts divided only in KM32 medium containing 1 mg/l 2,4-D and 0.5 mg/l BA. In addition, plating density was important because at densities below 5 x 10^4 protoplasts/ml, no divisions occurred whereas at 5 x 10^4 to 10^5, plating efficiencies up to 7.3% were achieved. For shoot regeneration, the protoplast colonies were moved to semi-solid media when they had grown to 0.5 mm in diameter. The regeneration medium contained 2iP, kinetin, BA, or zeatin at 0.1 or 1.0 mg/l in combination with 0.1 mg/l NAA. All of the cytokinins induced shoots in about 30% of the calluses depending on the cytokinin concentration. However, the authors did observe differences in shoot growth depending on

the cytokinin. BA produced slow growing dark green ferns whereas zeatin and 2iP promoted thin elongated ferns. Eight protoplast-derived plants were found to be aneuploid with 2n = 2x = 22 - 38 chromosomes.

Dan and Stephens (1991) developed a protoplast culture system specifically for *A. officinalis* L. cv. Lucullus 234 that has resistance to fusarium (Stephens et al. 1989). They tested the effects of three culture methods (liquid, agarose layer, and bead cultures), growth regulators and osmoticum on plating efficiency and colony formation. In the first experiment, they found that more divisions occurred when protoplasts were embedded in agarose versus liquid medium, and that KM medium containing 0.5 mg/l NAA, 0.5 mg/l 2,4-D and 0.5 mg/l kinetin was superior to other PGR combinations. Reducing the osmoticum from 800 mOsmol/kg to 360 increased the plating efficiency from 1.08 to 19.12%. Colony formation was also well supported at the lower osmoticum. They suggested that maintaining a high osmoticum may impair protoplast growth and metabolism and that the agarose protects the fragile cells and permits a lower osmoticum to be used. Following this, they re-evaluated the effect of PGR's on plating efficiency using protoplasts embedded in agarose beads in the lower osmotic medium. NAA, 2,4-D and kinetin were used in concert at 0.25, 0.5 or 1.0 mg/l, and the plating efficiency reached 19% with all PGR's at 0.5 mg/l. The protoplast-derived calluses were transferred to shoot regeneration medium containing various

combinations of NAA, 2,4-D, kinetin, zeatin and BA for four weeks and then to medium lacking hormones until shoots developed. The most effective medium contained 0.25 mg/l BA, 0.125 mg/l NAA and 0.125 mg/l 2,4-D and induced shoots in 92% of the calluses tested. Twenty eight plants were transferred to the greenhouse and 20 survived.

The studies by Elmer et al.(1989) and Dan and Stephens(1991) regenerated plants from protoplast-derived calluses via adventitious shoot formation. The following reports contrast those where plants were regenerated via somatic embryogenesis. Kunitake and Mii (1990) isolated protoplasts from embryogenic callus that had been initiated from spear sections of *A. officinalis* L. cv. Mary Washington on 1 mg/l 2,4-D. They found that they could dramatically increase the viability and protoplast yield by pretreating the callus for 4-7 days in MS medium lacking PGR's. The viability of the pretreated protoplasts was greater than 95% as determined by staining with fluorescein diacetate (FDA). The protoplasts were adjusted to 1 x 10^5 /ml in MS medium containing several combinations of NAA, 2,4-D, BA and zeatin, different osmotic agents, with or without 1000 mg/l glutamine and solidified with 0.1 Gellan gum. They found the most beneficial combination of factors for cell divisions of the embryogenic protoplasts was 1 mg/l NAA, 0.5 mg/l zeatin, 1000 mg/l glutamine and 0.6 M glucose as the sole osmoticum. This medium produced a plating efficiency of over 7% whereas any

other combination did not yield PE's above 0.5%. After 40-50 days of culture, the protoplast-derived colonies were placed on ½ MS medium with 1% sucrose and lacking PGR's. After about two weeks friable callus composed of various stages of embryos developed. Pretreating the embryos in distilled water for one week was critical for germination which occurred on ½ MS medium with 1 mg/l IBA, 1 mg/l GA_3, 1% sucrose and 0.2% Gellan gum. Embryos that were not pretreated rarely germinated. Twelve plants were moved to the greenhouse and all had 2n = 2x = 20 chromosomes.

Mukhopadhyay and Desjardins (1994a; 1994b) wanted to develop a simplified and reliable protocol for culturing embryogenic protoplasts from two genotypes of asparagus and regenerate plants via protoplast-derived somatic embryos. Similar to other studies, they investigated the effects of culture media, culture modes, plating densities and carbon sources on plating efficiency. Embryogenic callus, used as the source of protoplasts, was initiated from young spears of *A. officinalis* genotypes G203 and G171 on MS medium with 1 mg/l 2,4-D. The callus was subcultured every four weeks until a friable callus containing various stages of embryos developed. Their first experiment investigated the plating efficiency of G203 and G171 protoplasts cultured in liquid or semisolid (0.1% Gelrite) KM medium or ½ strength MS medium with 1 mg/l 2,4-D and 0.5 mg/l BA or 1 mg/l NAA and 0.5 mg/l zeatin. KM medium failed to produce plating efficiencies

above 0.25% for either genotype whereas MS medium produced PE's from 2.3 to 12.5%, and 1 mg/l NAA and 0.5 mg/l zeatin was more beneficial than 1 mg/l 2,4-D and 0.5 mg/l BA. As observed in other studies, the immobilized protoplasts (0.1% Gelrite) had a significantly higher PE than those in liquid culture only. They also observed consistently higher PE's with genotype G203 than G171. In a second experiment, they compared the effect of inoculum density and osmoticum on PE with both genotypes. The protoplast densities for this study, 10^4, 5 x 10^4, 10^5, and 2 x 10^5 and the osmoticum carbohydrates and levels, 0.1 M sucrose, 0.6 M glucose, and 0.7 M mannitol were used because they were optimal in other asparagus protoplast studies (Kong and Chin 1988; Elmer et al. 1989; Kunitake and Mii 1990; Mukhopadhyay and Desjardins 1994). Similar to the other reports, they found the optimal protoplast culture density was 1 x 10^5 (Elmer et al. 1989) and the highest PE was in protoplasts cultured with 0.6 M glucose as the osmoticum (Kunitake and Mii 1990). Genotype G203 again produced higher PE's than G171 in each treatment. The protoplast-derived calluses were placed on semi-solid MS medium with 1 mg/l 2,4-D and rapidly grew into friable and nodular calluses of an embryogenic nature. After four weeks, the embryogenic callus of G203 was subcultured to ½ MS medium lacking PGR's with 1% sucrose for embryo development. Of the embryos that developed, approx. 40% showed normal development and when those embryos were transferred to ½ MS medium

containing 1 mg/l GA_3, 1% sucrose and 0.2 % Gelrite, normal roots and shoots developed. Cytology was performed on 10 plants and each was found to have 2n = 2x = 20 chromosomes. Plant regeneration for genotype G171 was not mentioned.

Hsu et al. (1990) investigated several parameters for protoplast isolation and culture from asparagus suspension cultures since suspensions can provide large uniform populations of cells. Suspension cultures were initiated from anther-derived callus of *A. officinalis* cv. UC New Dwarf 5. One month-old callus was macerated and placed in 25 ml of liquid MS medium with 2 mg/l 2,4-D and agitated at 150 rpm under dim light. The suspensions were subcultured weekly by placing 5 ml of culture into 20 ml of fresh medium. Protoplasts were isolated 2, 4, 6 and 8 days after the 12th subculture to determine the effect of culture age on protoplast yield. Yield was greatest at 2 and 4 days with 42% and 50% of the cells yielding protoplasts, respectively. However, yield dropped off dramatically at 6 and 8 days with 15% and 5.8% yields, respectively. The reduction in yield may have been due to the presence of secondary cell walls that form as cell growth slows and are consequently more difficult to digest. Optimal protoplast yield was obtained at 4 days post subculture for up to 24 generations, although PE decreased with the age of the culture. They also found that protoplast yield was more than double when substituting glucose for mannitol as the osmoticum in the digestion

solution. Protoplasts were cultured at 1 x 10^5/ml in liquid KM medium. When colonies reached 0.1 to 0.3 mm in dia. they were placed on semi-solid MS medium with 2 mg/l 2,4-D until a friable embryogenic callus was produced. Embryos were then transferred to MS medium with 0.3 mg/l NAA and 0.1 mg/l kinetin for germination to plants.

Two similar studies examined the use of a polypropylene membrane as a support system to enhance asparagus protoplast culture (Kong and Chin 1988; Chin et al. 1988). Protoplasts were isolated from 5-6 day-old cell suspension cultures initiated from asparagus seedling-derived callus. The choice of osmoticum in the digestion solution affected speed in which first divisions occurred. Protoplasts digested in glucose divided faster than when digested in mannitol or sorbitol. They also found that the method of culture significantly affected divisions. Protoplasts failed to divide in liquid droplets alone although when the droplets were placed on the membrane floating on liquid medium, a plating efficiency of 1-5% was observed. Protoplasts embedded in agarose droplets produced around 1% divisions and the plating efficiency rose to at least 10% when the agarose was placed on the membrane system. The highest plating efficiency was achieved at 1 x 10^5 protoplasts/ml. The authors stated an advantage of using a porous membrane, besides enhancing cell division from protoplasts, is that the membrane can be easily handled and

placed on fresh medium without disturbing the protoplasts or developing colonies. A distinctive disadvantage is that the protoplasts can not be monitored with a microscope due to the membrane.

Transformation

Asparagus sp. are susceptible to infection by *Agrobacterium tumefaciens*. The lack of a wound response and the failure of the bacteria to attach to the plant cell wall are believed to be part of the reason why monocot species are recalcitrant to *Agrobacterium* transformation (Smith and Hood 1995). Asparagus, however, exhibits both of these requisites to transformation. The wound response in *A. officinalis* was studied using mechanically isolated mesophyll cells which are viable, damaged in a uniform manner and available in large numbers (Harikrishna et al. 1991). Two days after isolation, the cells underwent dramatic changes. The cells began to expand and divide by day five, respiration increased along with RNA and DNA synthesis, and several novel peptides had appeared which were not present in the unwounded tissue. These cytological, physiological and molecular changes are all characteristic of a wound response.

Two studies documented the ability of *A. tumefaciens* to attach to asparagus mesophyll cells. Draper et al.(1983) examined several factors that effected attachment of *Agrobacterium* to isolated *A. officinalis* mesophyll cells.

Cell attachment was measured as the amount of plant cell aggregation or clumping that resulted from bacterial attachment. Increasing time, plant cell density and bacteria density all increased the rate of aggregation. There was virtually no effect on aggregation when the mesophyll cells were fixed with glutaraldehyde. However, when the cells were heat killed, the rate of aggregation decreased. This indicated that viability was not required for attachment and that the heat treatment probably altered or disrupted the surface receptor molecules. Several strains of *A. tumefaciens* were studied and all wild-type strains could aggregate the cells. Moreover, when the same strains were cured of their Ti-plasmids, aggregation still occurred. This confirmed that the genes responsible for cell attachment lie on the bacterial chromosome. Terouchi et al. (1990) compared the ability of *A. tumefaciens* to attach to dicot and monocot cells by SEM. The *Agrobacterium* was observed to attach to vinca, rice and asparagus cells at the same rate. Bacteria cured of their virulence plasmids attached at the same rate as wild-type strains confirming the results of the above study that the genes for cell attachment are chromosomal. Both studies concluded that bacterial attachment is not the limiting factor in *Agrobacterium* transformation of monocots or asparagus.

Asparagus officinalis was the first monocot plant in which hormone-independent and opine-producing crown gall tissue could be isolated. Hernalsteens et al. (1984)

inoculated young spear sections from seedling derived plants of *A. officinalis* cv. 'Roem van Brunswijk' with the oncogenic *A. tumefaciens* strain C58. After incubating the explants on 1 mg/l BA and NAA, and 500 mg/l cefotaxime for four weeks, proliferating calluses arising from the sites of inoculation were placed on PGR-free medium. Some of the isolated calluses continued to proliferate on this medium. The authors reported that obtaining hormone-independent callus was reproducible but the frequency of such occurrences was about 10% based on total explants inoculated. Also, that the youngest or most apical portion of the spears typically gave a positive response. Several of the hormone-autotrophic calluses were tested for the presence of opines. Each of the transformed cultures contained nopaline whereas the control cultures did not. This confirmed the first successful isolation of transgenic tissue in a monocot or asparagus.

Conner et al. (1988) investigated the ability of four oncogenic strains of *A. tumefaciens* to produce tumors on seventeen genotypes of *A. officinalis*. Of the four strains, C58, A722, A281 and A4T, only C58 was capable of causing small raised tumors to form at the wound sites. Of the seventeen genotypes tested, only three produced nopaline positive tumors when strain C58 was used exclusively. Similar to the report by Hernalsteens et al.(1984), tumors were only observed on the upper portions of young spears.

Opine positive tissues have been isolated from two additional species of asparagus following inoculation with *A. tumefaciens*. Stems of *A. sprengeri* Regal and *A. tetragonous* Bresler were wounded and inoculated with wild-type strain A208. Both species produced nopaline positive tumors (Suseelan et al. 1987).

Prinsen et al. (1990) investigated the levels of endogenous PGR's in hormone-autotrophic crown gall tissues of asparagus that had been transformed with wildtype *A. tumefaciens* strain C58. Genes *1* (*iaa*M) and *2* (*iaa*H) from oncogenic *A. tumefaciens* code for enzymes in the IAA biosynthesis pathway and gene *4* (*ipt*) catalyzes the first step in cytokinin biosynthesis. The presence of these genes in hormone-autotrophic callus was confirmed by Southern hybridization. In regard to auxin biosynthesis, there was virtually no enhanced IAA levels in the tumor cell lines compared to the untransformed callus. However, there was a significant increase in endogenous cytokinins detected. This indicated that hormone-autotrophic growth of asparagus crown gall cells is only dependent upon an active gene *4* for increased cytokinin levels.

The first molecular evidence of T-DNA integration into a monocot plant was in *A. officinalis* (Bytebier *et al.* 1987). Hormone autotrophic tissues were produced as described by Hernalsteens *et al.* (1984) via transformation with oncogenic *A. tumefaciens* strain C58. Southern analysis of one of the

tumor tissues using several restriction enzymes and four probes spanning the T-DNA region, showed that the T-DNA was integrated into the asparagus genome and that no detectable deletions resulted from transfer or integration. Hence, the authors concluded that T-DNA could be stably integrated into monocots (asparagus) and that the T-DNA in asparagus was identical to integration observed in dicots. In the second part of this study, the first reported transgenic asparagus plants transformed via *Agrobacterium* were produced and analyzed. Young spear sections were inoculated with the non-oncogenic *A. tumefaciens* strain C58C1 containing pGV3850::1103neo. This cointegrated vector possessed the NPTII gene for kanamycin resistance and the nopaline synthase gene. After one month, 2 mm slices of infected tissue were cultured on LS medium with 1 mg/l BA and 1 mg/l NAA and 200 mg/l glutamine. The explants were subcultured to the same medium after a month for one additional month of culture. The tissue was then transferred to the same medium containing 50 mg/l kanamycin. Three of 25 calluses isolated continued to grow on kanamycin containing medium. Nopaline was detected in each of the kanamycin resistant tissues. Two of the three resistant cell lines gave rise to transgenic plants after being subcultured to medium containing 40 mg/l adenine, 4 mg/l BA and 1 mg/l NAA. Southern analysis of three plants from the same callus line showed integration of the T-DNA into high-molecular weight DNA and that no major rearrangements occurred

during regeneration. The authors concluded that T-DNA integration in asparagus is comparable to that in dicots.

Conner et al. (1988) tested the ability of four strains of wild-type *A. tumefaciens* to produce tumors on 17 genotypes of *A. officinalis*. From this study they determined that *Agrobacterium* strain C58 produced the most tumors on asparagus genotype CRD 157. Based on these data, they attempted to produce transgenic plants of genotype CRD157 using the non-oncogenic version of the same strain, C58C1, containing pGV3850::1103neo. The cointegrate vector possessed both the NPTII and nopaline synthase genes. *In vitro* explants of CRD157 were inoculated with the bacteria and cocultivated on callusing medium (MS medium containing 200 mg/l glutamine, 1 mg/l NAA and 1 mg/l kinetin) for 2-3 days. Subsequently, the cultures were placed on the same medium containing 250 mg/l cefotaxime to prevent bacteria growth. Kanamycin selection (100 mg/l) was imposed after 10 days of culture. After seven months of culture on kanamycin, 3 of 125 explants produced resistant calluses. All three of the cell lines produced nopaline. Two of the cell lines failed to continue to grow beyond five subcultures and the third one maintained stable kanamycin resistance and nopaline synthesis for over 18 months. Transgenic plants were regenerated from this last cell line and Southern hybridization confirmed the integration of the NPTII gene.

Long-term embryogenic callus of *A. officinalis* was used as a target for *Agrobacterium*-mediated transformation to determine if these cultures could be efficiently used to produce transgenic material (Delbreil et al. 1993). Three long-term embryogenic cultures, L1, L2 and L3, were established from three male genotypes as described by Delbreil et al. (1994). The cultures were inoculated with *A. tumefaciens* strain C58 containing p35SGUSINT. The binary vector possesses the NPTII gene for kanamycin resistance and an intron containing GUS gene that can only be expressed in the plant cell. One gram of somatic embryos were inoculated in 5 ml of OD_{600} 0.6 - 0.8 bacteria suspension for 15 minutes. The embryos were then blotted dry, placed on B medium for cocultivation and incubated in darkness for 48 hr. Following cocultivation, the embryos were subcultured to B selection medium containing 400 mg/l cefotaxime and 100 mg/l kanamycin. After two to three months on selection medium, resistant embryogenic lines began to develop from necrosed primary embryos. A total of 23 resistant cultures were isolated from the three original cell lines. Lines L1, L2, and L3 produced 14, 8 and 1 resistant line(s), respectively. The transformation frequencies achieved for each long-term embryogenic line based on resistant cell lines isolated per gram of embryos inoculated was 0.6, 4 and 1 for L1, L2 and L3, respectively. Only three of the kanamycin resistant lines from the L1 culture gave rise to plantlets out of all the

resistant lines isolated. All resistant cell lines and the resulting plantlets were positive for GUS expression. Embryo stage, cocultivation medium and timing of selection were compared in an effort to increase the transformation efficiency. The results showed that globular embryos were resistant to transformation compared to elongated embryos, solid medium was superior to liquid during cocultivation and that applying selection pressure within seven days after cocultivation was beneficial. Southern analysis confirmed the stable integration of the NPTII and GUS genes in four resistant cell lines and the integration of the NPTII gene in one transgenic asparagus plant. One and two sites of T-DNA integration were identified in the material tested similar to integration patterns found in dicot plants transformed with *Agrobacterium*. The overall transformation frequency was very low although the authors did not consider this a limiting factor. Since each gram of material contained about 300 embryos, the low transformation frequency could be compensated for by increasing the amount of material inoculated.

There has been one report of direct-gene-mediated transformation in asparagus (Mukhopadhyay and Desjardins 1994). Factors influencing transient and stable GUS expression following electroporation of plasmid DNA into callus-derived protoplasts were studied. Field strengths at 500 and 750 V/cm were optimal for transient expression but reduced protoplast viability. Both heat shock and the

addition of polyethylene glycol to the electroporation solution enhanced transient and stable GUS expression. Two genotypes were tested and G203 outperformed G171 under each experimental parameter. After the electroporated protoplasts had been cultured for 28 days, the colonies were subcultured to 50 mg/l kanamycin. Kanamycin resistant calluses developed within 2 - 3 weeks and were tested for stable GUS expression. Each one of the resistant colonies was also GUS positive. Although GUS positive/kanamycin resistant colonies were obtained, regeneration of transgenic plants was not reported.

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Chapter Two

Genotype and auxin influence direct somatic embryogenesis from protoplasts derived from embryogenic cell suspensions of *Asparagus officinalis* L.

ABSTRACT

Embryogenic callus from four asparagus genotypes, Jersey Giant No. 8, MD10, Rutgers 22, and 86SOM1 was simultaneously initiated from spear explants on semisolid LS medium containing 5 µM 2,4-D or 50 µM NAA. Calluses were used to initiate cell suspensions in liquid LS medium of the same composition. The eight sets of cell suspensions were used as protoplast donors at both two and five months of age. Protoplasts were immobilized at 10^5/ml density in MS medium with 0.6% agarose and overlaid with liquid KM medium; both containing the same type and concentration of auxin used for the corresponding donor cells or with plant growth regulator-free (PGR-free) medium. There was a significant interaction between genotype, suspension auxin, and inclusion or exclusion of PGRs in the protoplast culture media on plating efficiency, and colony and somatic embryo formation. Plating efficiencies at 14 days ranged from 0 - 40%. Globular somatic embryos developed directly from protoplasts in 10 - 14 days and bipolar embryos could be transferred in 3 - 4 weeks to embryo maturation medium (EM medium) composed of LS medium with 2% sucrose and 1% Phytagel. Conversion to plants occurred as rapidly as 1 - 2 weeks after transfer to EM medium or 5 - 6

weeks after initial protoplast culture. Although all four genotypes regenerated plants, Rutgers 22 had the highest conversion frequency at 42%. Most plants recovered from the 2,4-D-derived protoplasts were karyotypically aberrant while a higher frequency of normal plants were obtained from the NAA-derived protoplast cultures.

Introduction

Asparagus, (*Asparagus officinalis* L.), is an economically important crop cultivated throughout the world for its edible spears (Nichols 1990). Genetic improvement of asparagus by somatic hybridization and transformation via direct-gene-transfer have been suggested as applications of asparagus protoplast regeneration systems (Bui Dang Ha 1975; Kong and Chin 1988; Elmer *et al.* 1989; Hsu *et al.* 1990; Kunitake and Mii 1990). Although protoplast to whole plant schemes have been reported, there is still a lack of consensus on the optimum auxin type for culture of donor tissues and protoplasts.

The choice of auxin is important for donor tissue and subsequent protoplast culture; particularly in monocots. Both 2,4-D and NAA have been used to maintain asparagus callus and suspension cultures as protoplast donors; however, the best plant growth regulator (PGR) for donor cells is not necessarily the same as that for protoplast culture. Moreover, 2,4-D and NAA have both been shown to be optimal for asparagus protoplast culture alone or in combination (Bui Dang Ha and Mackenzie 1973; Chin *et al.* 1988; Kong and Chin 1988; Elmer *et al.* 1989; Hsu *et al.* 1990; Kunitake and Mii 1990). Such inconsistencies may be confounded by using different genotypes to develop each culture system.

Asparagus is a heterozygous dioecious crop; therefore, each individual is a distinct genotype which may vary from

others in *in vitro* responses (Tsay *et al.* 1981). Genotype effects in asparagus have been observed in anther culture (Tsay *et al.* 1981), micropropagation (Slimmon *et al.* 1985) and rooting of micropropagules (Jamieson *et al.* 1985). Plant regeneration from protoplasts can also be strongly dependent on genotype (Roest and Gilissen 1993). Previous work from our laboratory indicated that only one of four genotypes studied (Jersey Giant No. 8), yielded protoplasts capable of sustained divisions and regenerated plants (Elmer *et al.* 1989). Other protoplast studies have used either a single genotype (Bui Dang Ha and Mackenzie 1973; Hsu *et al.* 1990; Dan and Stephens, 1991) or seed propagated varieties (Chin *et al.* 1988; Kong and Chin 1988; Kunitake and Mii 1990).

Somatic embryogenesis was chosen in this study as the mode of regeneration because both shoot and root apices are present in mature somatic embryos which can expedite crown formation (Levi and Sink 1991). In contrast, rooting of asparagus shoots derived from organogenesis can be difficult and genotype dependent (Jamieson *et al.* 1985).

In this study, the effect of auxin and genotype on sequential developmental steps of direct somatic embryogenesis from protoplasts was investigated using 2,4-D and NAA-derived embryogenic cell suspensions of four genotypes of *A. officinalis*.

Materials and Methods

Plant material

Four genotypes of *Asparagus officinalis* L., Jersey Giant No. 8 (JG 8), 86SOM1, MD10, and Rutgers 22, were micropropagated according to Slimmon *et al.*(1985). Rutgers 22 is a male that has generated supermales for breeding purposes. Jersey Giant No. 8 was used as a positive control because it responded well to protoplast culture in a previous study in our lab (Elmer *et al.* 1989). 86SOM1 is an outstanding clone male clone that was selected in a Michigan asparagus field and is currently bein evaluated for potential release. MD10 is an outstanding female clone and potential breeding parent.

Establishment and maintenance of callus and suspension cultures

Elongating shoots from micropropagated crowns of each genotype were cut transversely into 1 - 2 cm explants, each possessing lateral buds, and placed on Linsmaier and Skoog (LS) medium (1965), with 2% sucrose, 0.8% Bacto agar (pH 5.8) and either 5 µM 2,4-D or 50 µM NAA. Cultures were incubated at 26°C, and 25 $\mu molm^{-2}s^{-1}$ light supplied by Philips F96T12/CW cool white bulbs on a 16-h photoperiod. Callus originating from the lateral buds was subcultured monthly on the same medium. After three subcultures, yellowish-white embryogenic callus possessing immature somatic embryos that formed at the

surface of mucilaginous callus was used to initiate cell suspensions.

Approximately 250 mg of callus of each genotype from either 2,4-D or NAA induced cultures was inoculated into each of four 250 ml Erlenmeyer flasks each containing 40 ml of the same medium used to initiate the callus. The eight sets of cultures were incubated at 26°C, under 4 $\mu molm^{-2} s^{-1}$ illumination for 16 h on a gyratory shaker at 110 rev./min. Subcultures were performed weekly by sieving cells through 1 mm polyester mesh and transferring ~0.5 ml of cells, settled cell volume (SCV), to 40 ml fresh medium. The suspensions were maintained on a pedigree basis for the first month and then one of the four cultures from each treatment was visually selected for further subculture based on the presence of pro-embryogenic masses (PEMs) and clusters of globular somatic embryos.

Isolation of protoplasts

Protoplasts were isolated from 2-month-old embryogenic suspensions and again at five months from the same cultures. Suspension cells, five days after subculture, were washed with LS medium plus 2% sucrose and digested by incubating 1 g of cells in 15 ml of filter-sterilized enzyme solution containing 1% Cellulysin, 0.2% Macerase (Calbiochem), 1% Rhozyme (Rohm and Haas Co.), 5 mM MES and 0.6 M glucose, pH 5.8 in CPW salts (Frearson *et al.* 1973). Cells were digested for 16 h in darkness at 25°C and 25 rev./min on a gyratory shaker.

Following digestion, protoplasts were sieved through 61 µm nylon mesh, collected, centrifuged (50× *g;* 5 min) and the pellets washed once by resuspending in wash medium (0.6 M glucose in CPW salts, pH 5.8) and recentrifuging (50× *g;* 5 min). Protoplasts were purified by resuspending the pellets in 9 ml of 21% sucrose in CPW salts (pH 5.8) overlaid with 1 ml wash medium and centrifuging (150× *g;* 10 min). Protoplasts were collected from the media interface, resuspended in 10 ml of wash medium and counted.

Culture of protoplasts

Protoplasts were suspended at 1 x 10^5/ml in molten (35°C) Murashige and Skoog medium (MS)(1962) with 1 g/l glutamine, 0.6 M glucose, and 0.6% agarose (Seaplaque, FMC) (pH 5.8). Fifteen 25 µl agarose discs containing the suspended protoplasts were placed in one quadrant of a 100 x 15 mm X-plate with the two opposing quadrants containing 9 ml of modified reservoir medium lacking PGRs (Guri *et al.* 1987). The remaining quadrant was left empty. The agarose disks were allowed to solidify for 1 h at 23°C prior to the addition of the liquid medium. Six ml of filter-sterilized modified Kao and Michayluk medium (KM) (Kao and Michayluk, 1981) prepared according to Elmer *et al.*(1989) with the addition of 1 g/l glutamine, 2% (v/v) coconut water (Gibco) and 0.6 M glucose (pH 5.8) was overlaid on the agarose discs. Protoplasts derived from the 2,4-D or NAA cell suspensions were cultured

in MS agarose medium and overlaid with KM liquid medium both containing 5 µM 2,4-D or 50 µM NAA and 2 µM BA or lacking PGRs, respectively (see Table 1 for treatment combinations).

Cultures were incubated in darkness for 14 days and then transferred to 10 $\mu molm^{-2}s^{-1}$ light for seven days and then 25 $\mu molm^{-2}s^{-1}$ light for another seven days at 26°C, both on a 16-h photoperiod. The two experiments were arranged in 4 x 2 x 2 factorials with 4 genotypes, 2 donor culture auxins, and the presence or absence of PGRs in the protoplast media in a randomized complete block design with five replications. Data were collected at 14 days on plating efficiency (PE), colony formation per disc (≥8 cells/colony), and somatic embryos per X-plate. PE was recorded as the number of dividing cells/total number of protoplasts plated × 100. At 28 days, somatic embryos per disc and total colonies or embryos ≥0.5 mm per plate were recorded. Somatic embryos were classified as globular, elongated or mature bipolar structures possessing a defined protoderm or epidermis. For PE and colony formation at 14 days, and somatic embryos per disc at 28 days, five random agarose discs were sampled per X-plate. Data were analyzed by analysis of variance using MSTAT-C (version 1.2). Least significant differences were calculated at the 5% level of probability.

Germination of somatic embryos

At 28 days of culture, colonies and somatic embryos from each treatment were washed in LS medium with 2% sucrose and placed in 100 x 15 mm Petri plates containing embryo maturation medium (EM medium) consisting of LS medium with 2% sucrose and 1% Phytagel (Sigma) (medium 1.0G) (Saito *et al.* 1991). After four weeks, colonies and somatic embryos were transferred to GA-7 Magenta boxes each containing 60 ml of plantlet development medium (PDM) consisting of half-strength LS medium plus 2% sucrose and 0.8% agar for an additional four weeks. All cultures were incubated at 26°C and under 25 $\mu molm^{-2}s^{-1}$ illumination on a 16-h photoperiod. Ten colonies or somatic embryos were placed in one to five replications for the first experiment and 16 were placed in one to six replications for the second experiment or a maximum of 50 and 96 plated per treatment, respectively. Embryos were recorded as germinated if the radicle had elongated into a root and as converted if a root and shoot were both present at the end of eight weeks (Lai and McKersie 1993).

Comparison between conversion of somatic embryos from 5-month-old cell suspensions and their protoplast-derived somatic embryos

Cells from each of the suspensions used for protoplast isolation were washed with LS medium with 2% sucrose, sieved through 1 mm mesh and PEMs <1 mm were used for conversion studies. One-half ml of the SCV was resuspended in 50 ml of

the wash medium and 1 ml aliquots were pipetted into five 100 x 15 mm plates each containing 20 ml of EM medium. After four weeks, 16 somatic embryos from each plate were subcultured to five GA-7 boxes each containing 60 ml of PDM medium for an additional four weeks. Incubation conditions were identical to those for germination of protoplast-derived embryos. Treatments were arranged in a randomized complete block design with five replications. Paired and non-paired t-tests were performed on percent converted data for the protoplast-derived embryos from the 2,4-D - 2,4-D and NAA - NONE treatments and data for the cell suspension derived embryos using MSTAT-C.

Cytology

Chromosome numbers of root tip cells from protoplast-derived plants were determined according to Elmer et al.(1989). A minimum of two roots and 3 - 8 mitotic figures per root were analyzed per regenerant.

Results

Suspension cultures and protoplast yields

The suspension cultures had a heterogeneous cell composition at two months. Those of Rutgers 22 on 2,4-D and NAA, and 86SOM1 and MD10 on 2,4-D contained globular to torpedo stage embryos, single vacuolated cells, and PEMs composed of small cytoplasmic dense cells. Cultures of JG 8 on both 2,4-D and NAA were composed mostly of PEMs, and 86SOM1

and MD10 cultures in NAA had mostly colonies of large vacuolated cells that did not appear embryogenic. However, by the fifth month of culture, the suspensions became generally homogeneous and by the fifth month of culture all suspensions were composed mostly of PEMs.

Protoplast yields ranged from 0.3 to 10.1 × 10^6/gram cells and from 0.3 to 9.9 × 10^6/gram for 2-month-old and 5-month-old suspensions, respectively. Isolations from 2-month-old suspensions contained 20-40 µm cytoplasmic dense protoplasts with or without starch and up to 60-70 µm vacuolated protoplasts exhibiting cytoplasmically streaming depending on the culture. Five-month-old suspensions gave rise to more uniform populations of 20-50 µm cytoplasmically dense protoplasts. The yield of protoplasts from JG 8 NAA suspensions was considerably greater than all others at two and five months (10.1 and 9.9 × 10^6/gram, respectively), and most of the protoplasts were devoid of starch (Fig. 1A). In contrast, protoplasts of most other suspensions contained numerous starch grains (Fig. 1B).

Protoplast culture

Protoplasts from 2-month-old suspensions of Rutgers 22 and JG 8 were more responsive in culture than those from 86SOM1 and MD10 (Table 1). First divisions of NAA-derived protoplasts were observed at three days for Rutgers 22 and JG 8 cultured without PGRs {Rutgers 22 and JG 8 NAA protoplasts

Fig. 1. Direct somatic embryogenesis from embryogenic suspension-derived protoplasts of *Asparagus officinalis*. (A) Protoplasts devoid of starch derived from JG 8 NAA suspension cells. (B) Embryogenic suspension-derived protoplasts containing numerous starch grains. (C) A globular somatic embryo at 14 days developed directly from a Rutgers 22 NAA protoplast cultured in (-)PGR medium. Note the presence of a rudimentary suspensor (arrow). (D) Globular through mature somatic embryos at 28 days of culture developed directly from Rutgers 22 NAA protoplasts in (-)PGR medium. (E) Non-embryogenic protocolonies from JG 8 NAA protoplasts. (F) Converted protoplast-derived somatic embryos seven weeks after initial protoplast isolation. Bar = 100 µm.

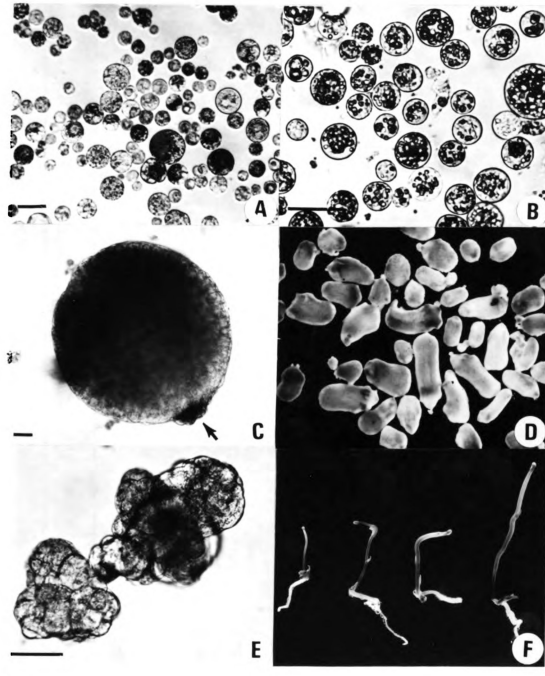
A
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Table 1. Effects of genotype, suspension media PGR, and protoplast media PGR on mean plating efficiency, colony and somatic embryo development from protoplasts derived from 2-month-old suspension cultures.

			14 days			28 days	
Genotype	Suspension PGR	Protoplast PGR	PE (%)	Colonies /disc	Embryos /plate	Colonies ≥0.5mm/plate	Embryos /disc
Rutgers 22	2,4-D	None	0	0	0	0.4 ± 0.2	0
	2,4-D	2,4-D	0	0	0	18.0 ± 3.6	0.2 ± 0.2
	NAA	None	7.2 ± 0.6	18.6 ± 2.0	11.4 ± 4.2	203.0 ± 9.1	22.9 ± 2.4
	NAA	NAA	1.0 ± 0.4	0.9 ± 0.7	0	14.2 ± 8.6	1.2 ± 1.0
JG 8	2,4-D	None	1.4 ± 0.3	1.2 ± 0.3	0.8 ± 0.4	32.4 ± 7.9	0.9 ± 0.2
	2,4-D	2,4-D	9.7 ± 1.5	7.8 ± 1.4	0.8 ± 0.4	55.0 ± 11.1	5.4 ± 0.6
	NAA	None	40.0 ± 1.2	111.5 ± 8.5	0.6 ± 0.4	174.2 ± 8.1	0
	NAA	NAA	0.1 ± 0.1	0.1 ± 0.1	0	0	0
86SOM1	2,4-D	None	0	0	0	0	0
	2,4-D	2,4-D	0	0	0	1.2 ± 0.8	0
	NAA	None	0.9 ± 0.1	1.0 ± 0.1	0	8.2 ± 1.3	0
	NAA	NAA	0.5 ± 0.3	0.6 ± 0.3	0	3.4 ± 1.9	0
MD10	2,4-D	None	0	0	0	0	0
	2,4-D	2,4-D	0.2 ± 0.1	0	0	7.6 ± 3.3	0.2 ± 0.2
	NAA	None	0.1 ± 0.1	0	0	0	0
	NAA	NAA	0.1 ± 0.1	0	0	0.4 ± 0.2	0
LSD (P = 0.05)			1.5	6.3	3.0	15.1	1.9

±, standard error.

(-)PGR}; whereas, first and second divisions in cultures of the other two genotypes occurred after 7 - 14 days. At 14 days, PE was highest at 40% for JG 8 NAA protoplasts (-)PGR followed by 9.7% for JG 8 2,4-D protoplasts cultured in the presence of PGRs {(+)PGR}, and 7.2% for Rutgers 22 NAA protoplasts (-)PGR (Table 1). All other cultures had low PEs of 0 to 1.4%. Mean colony formation (≥8 cells) per agarose disc was also highest, 111.5, for JG 8 NAA protoplasts (-) PGRs. In contrast, the same protoplasts cultured in the presence of 50 µM NAA virtually failed to divide. In addition to colonies present in the Rutgers 22 NAA (-)PGR cultures at 14 days, somatic embryos that had developed directly from protoplasts were observed. These embryos were 200-500 µm globular to early torpedo stage that possessed a smooth defined protoderm or epidermis that distinguished them from surrounding colonies (Fig. 1C). A few embryos were observed in other treatments at 14 days. At 28 days, Rutgers 22 NAA protoplasts (-)PGR had the highest number of somatic embryos per (agarose) disc that were mostly globular to mature bipolar embryos up to 5 mm in length (Fig. 1D). Both Rutgers 22 and JG 8 NAA (-)PGR cultures at 28 days had the greatest number of colonies and/or somatic embryos ≥0.5 mm. Whereas Rutgers 22 produced mostly somatic embryos, the JG 8 culture produced primary cell colonies devoid of organized structures (Fig. 1E).

As previously mentioned, the suspensions became more homogeneous by the fifth month of culture. This uniformity probably allowed protoplasts from 86SOM1 and MD10 to respond to a significant degree along with Rutgers 22 and JG 8 (Table 2). PE at 14 days was highest at 30.6% from protoplasts of JG 8 NAA (-)PGR, and at 11.8% for Rutgers 22 NAA (-)PGR. The former decreased from 40% and the latter increased from 7.2% as compared to those from 2-month-old suspensions.

A significant three way interaction was observed between genotype, suspension PGR and presence or absence of PGRs on PE (ANOVA not shown). PE was highest for Rutgers 22, JG 8 and MD10 protoplasts from NAA suspensions cultured in the absence of PGRs, and for 86SOM1 from 2,4-D suspension-derived protoplasts cultured in the presence of PGRs (Table 2). Disregarding the effect of PGRs in the protoplast media, a significant interaction between genotype and auxin was evident on PE since the greatest response for each genotype was dependent on the auxin used to initiate and maintain the donor cells. A trend was apparent at 14 days in that if protoplasts were derived from 2,4-D cell suspensions, significantly higher PE and colony formation was achieved when 5 µM 2,4-D was included in the protoplast culture medium with the exception of MD10. Conversely, the addition of 50 µM NAA to the NAA-derived protoplasts was inhibitory and even appeared toxic with the exception of 86SOM1 which did not respond with or without PGRs (Table 2).

Table 2. Effects of genotype, suspension media PGR, and protoplast media PGR on mean plating efficiency, colony and somatic embryo development from protoplasts derived from 5-month-old suspension cultures.

			14 days			28 days	
Genotype	Suspension PGR	Protoplast PGR	PE (%)	Colonies /disc	Embryos /plate	Colonies ≥0.5mm/plate	Embryos /disc
Rutgers 22	2,4-D	None	0.1 ± 0.1	0.2 ± 0.1	0	1.2 ± 0.8	0.2 ± 0.2
	2,4-D	2,4-D	1.3 ± 0.4	3.4 ± 1.2	0	9.2 ± 1.4	0.5 ± 0.2
	NAA	None	11.8 ± 0.7	15.5 ± 0.9	2.4 ± 0.6	133.2 ± 8.0	15.1 ± 0.5
	NAA	NAA	0	0	0	0	0
JG 8	2,4-D	None	1.0 ± 0.2	0.2 ± 0.1	0	1.6 ± 0.8	0.1 ± 0.1
	2,4-D	2,4-D	5.4 ± 0.7	2.8 ± 0.8	0	8.6 ± 1.5	0.6 ± 0.1
	NAA	None	30.6 ± 0.4	92.8 ± 1.9	0	151.8 ± 8.3	0
	NAA	NAA	0	0	0	0	0
86SOM1	2,4-D	None	2.0 ± 0.4	3.4 ± 0.4	0	62.0 ± 6.8	2.2 ± 0.4
	2,4-D	2,4-D	8.9 ± 0.7	9.1 ± 1.3	0	110.0 ± 8.5	1.2 ± 0.4
	NAA	None	0.3 ± 0.2	0.2 ± 0.2	0	3.0 ± 0.6	0.1 ± 0.1
	NAA	NAA	0	0	0	0	0
MD10	2,4-D	None	0	0	0	0.6 ± 0.4	0.1 ± 0.1
	2,4-D	2,4-D	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	9.0 ± 3.2	0.5 ± 0.3
	NAA	None	6.9 ± 0.5	18.9 ± 2.0	3.4 ± 1.2	208.2 ± 8.3	17.0 ± 0.8
	NAA	NAA	0.2 ± 0.1	0.2 ± 0.0	0	7.6 ± 2.2	0
LSD (P = 0.05)			1.1	2.5	0.9	12.7	0.8

±, standard error.

Direct somatic embryo development from 5-month-old suspension-derived protoplasts was observed at 14 days for Rutgers 22 and MD10 cultures and for all four genotypes at 28 days (Table 2). Fewer embryos per plate (2.4) were observed at 14 days from Rutgers 22 NAA (-)PGR compared to the same treatment from 2-month-old suspensions (11.4/plate). Direct embryogenesis was also observed at 14 days in MD10 NAA (-)PGR cultures which was not evident from the 2-month-old suspension-derived protoplasts. Mean embryos per disc at 28 days was fairly low for JG 8 and 86SOM1, 0.6 and 2.2, respectively, and occurred almost exclusively from 2,4-D-derived protoplasts (Table 2). Significantly more embryos developed from Rutgers 22 and MD10 with the greatest number of embryos per disc occurring from NAA- derived protoplasts cultured in the absence of PGRs. Protoplasts from all genotypes exhibited sustained divisions and gave rise to numerous colonies and/or embryos ≥0.5 mm within 28 days.

Plant regeneration

Plant regeneration via somatic embryogenesis was achieved for three of the four genotypes from the 2-month-old suspension derived protoplasts (Table 3). Embryo conversion was greatest from Rutgers 22 NAA (-)PGR and (+)PGR treatments with 42% and 35% forming plantlets, respectively, followed by 26% from MD10 2,4-D (+)PGR. None of the embryos in these three treatments exhibited callus formation. In contrast,

Table 3. Germination and converstion to plants of protoplast-derived somatic embryos from 2-month-old suspension cultures.

Genotype	Suspension PGR	Protoplast PGR	N[a]	Calli	Germinated somatic embryos: Root only	Germinated somatic embryos: Root and shoot	Conversion (%)
Rutgers 22	2,4-D	None	2	2	0	0	
	2,4-D	2,4-D	50	43	4	1	2 ± 2
	NAA	None	50	0	20	21	42 ± 7
	NAA	NAA	40	0	19	14	35 ± 7
JG 8	2,4-D	None	50	50	0	0	
	2,4-D	2,4-D	50	49	0	1	2 ± 2
	NAA	None	50	50	0	0	
	NAA	NAA	0				
86SOM1	2,4-D	None	0				
	2,4-D	2,4-D	8	0	6	0	
	NAA	None	40	22	8	0	
	NAA	NAA	30	15	11	0	
MD10	2,4-D	None	0				
	2,4-D	2,4-D	50	0	15	13	26 ± 6
	NAA	None	0				
	NAA	NAA	2	1	0	1	50

±, standard error.

[a]Number of colonies or somatic embryos with a maximum of ten plated per replication.

cultures such as JG 8 and 86SOM1 had a high degree of callus formation and low embryo conversion (Table 3). Plant regeneration occurred for each genotype from protoplasts derived from 5-month-old suspensions, however, conversion was lower overall compared to those of the 2-month-old cultures (Table 4). Conversion of Rutgers 22 NAA (-)PGR embryos decreased from 42% to 19%, and from 26% to 19% for MD10 2,4-D (+)PGR. Although JG 8 NAA (-)PGR protoplasts had the highest PE for both 2- and 5-month-old suspensions, mature embryos were not produced.

Some protoplast-derived somatic embryos from Rutgers 22 and MD10 converted into plantlets as rapidly as two weeks after subculture to EM medium (Fig. 1F). However, the majority of embryos required a longer time to mature (4 - 5 wks) due primarily to the presence of less developed stages at the end of protoplast culture. These embryos subsequently germinated upon subculture to PDM medium and could be transferred to the greenhouse as rapidly as three months after initial protoplast isolation.

Comparison of embryo conversion from donor cell suspensions and their derived protoplasts

Conversion frequency was greater for each genotype for the suspension cell-derived embryos; however, Rutgers 22 NAA and 2,4-D, and MD10 2,4-D cultures were not significantly different from the protoplast-derived cultures (Table 5).

Table 4. Germination and conversion to plants of protoplast-derived somatic embryos from 5-month-old suspension cultures.

Genotype	Suspension PGR	Protoplast PGR	N[a]	Calli	Germinated somatic embryos: Root only	Germinated somatic embryos: Root and shoot	Conversion (%)
Rutgers 22	2,4-D	None	16	0	10	1	6
	2,4-D	2,4-D	96	18	39	9	9 ± 1
	NAA	None	96	6	43	18	19 ± 4
	NAA	NAA	0				
JG 8	2,4-D	None	16	16	0	0	
	2,4-D	2,4-D	80	34	28	7	9 ± 3
	NAA	None	96	96	0	0	
	NAA	NAA	0				
86SOM1	2,4-D	None	96	83	5	0	
	2,4-D	2,4-D	96	23	38	4	4 ± 2
	NAA	None	32	32	0	0	
	NAA	NAA	0				
MD10	2,4-D	None	4	0	2	1	25
	2,4-D	2,4-D	48	0	23	9	19 ± 4
	NAA	None	96	64	12	8	8 ± 4
	NAA	NAA	16	14	0	0	

±, standard error.

[a]Number of colonies or somatic embryos with a maximum of 16 plated per replication.

Both 86SOM1 2,4-D and NAA suspension cells had higher embryo conversion than from protoplasts. JG 8 2,4-D and MD10 NAA suspensions had significantly more embryos that converted into plantlets than from their protoplasts. The most notable difference was observed between MD10 NAA cultures with suspension-derived embryos converting at 58% compared to 8% for protoplast-derived embryos. Protoplasts from JG 8 failed to regenerate plants and the donor suspensions also proved to be non-embryogenic.

Table 5. Comparison of conversion frequency of somatic embryos from 5-month-old suspension culture cells with their derived protoplasts.

			Protoplast derived		Suspension derived		
Genotype	Suspension PGR	Protoplast PGR	*N*	Conversion (%)	*N*	Conversion (%)	*t*-test
Rutgers 22	2,4-D	2,4-D	96	9 ± 1	80	13 ± 4	ns
	NAA	None	96	19 ± 4	80	21 ± 3	ns
JG 8	2,4-D	2,4-D	80	9 ± 3	80	33 ± 4	**
	NAA	None	96	0	0	0	
86SOM1	2,4-D	2,4-D	96	4 ± 2	80	13 ± 3	*
	NAA	None	32	0	80	10 ± 3	
MD10	2,4-D	2,4-D	48	19 ± 4	80	20 ± 4	ns
	NAA	None	96	8 ± 4	80	58 ± 7	**

NS, *, ** Significant at $P > 0.05$, and $P < 0.05$ and 0.01, respectively.
±, standard error.

Cytology

Cytology was performed on a maximum of five protoplast derived-plants from each culture-regeneration treatment (Table 6). In general, plants regenerated from NAA cultures had a higher frequency of normal karyotypes (2n=20) than those from

Table 6. Chromosome numbers of protoclones derived from 2- and 5-month-old suspension cultures.[a]

2-month-old suspensions				5-month-old suspensions			
Regenerant	Suspension PGR	Protoplast PGR	Chromosome no.	Regenerant	Suspension PGR	Protoplast PGR	Chromosome no.
Rutgers 22-1	2,4-D	None	42-70	Rutgers 22-1	2,4-D	None	60-70
Rutgers 22-1	NAA	None	34	Rutgers 22-1	2,4-D	2,4-D	40-70
Rutgers 22-2			20	Rutgers 22-2			33-41
Rutgers 22-3			20	Rutgers 22-3			35
Rutgers 22-4			20	Rutgers 22-4			30-72
Rutgers 22-1	NAA	NAA	20	Rutgers 22-5			37
Rutgers 22-2	NAA	NAA	20	Rutgers 22-1	NAA	None	30
JG 8-1	2,4-D	2,4-D	28	Rutgers 22-2			30
MD10-1	2,4-D	2,4-D	24-66	Rutgers 22-3			20
MD10-2			63-72	Rutgers 22-4			20
MD10-3			24-70	Rutgers 22-5			20
MD10-4			42-80	JG 8-1	2,4-D	2,4-D	32
MD10-5			50-60	JG 8-2			36
MD10-1	NAA	NAA	20	JG 8-3			60
				JG 8-4			36
				86SOM1-1	2,4-D	2,4-D	32
				86SOM1-2			36
				86SOM1-3			60
				86SOM1-4			20
				86SOM1-5			20
				MD10-1	2,4-D	2,4-D	70
				MD10-2			60
				MD10-3			37-51
				MD10-4			40
				MD10-5			40
				MD10-1	NAA	None	37
				MD10-2			20
				MD10-3			20

[a]Normal karyotype is 2n = 20.

2,4-D cultures. All plants except one (86SOM1-5) derived from protoplasts of 2,4-D suspensions were aneuploid, polyploid or mixoploid. Culture age did not appear to be an influence since cytogenetically normal and aberrant plants were regenerated from the same donor suspensions at two or five months.

Phenotypic differences between regenerated plants from 2,4-D or NAA derived protoplasts were observed *in vitro* primarily in regenerants of MD10. MD10 NAA-derived plants were vigorous in both shoot and root production compared to 2,4-D-derived plants that produced short thickened shoots that elongated poorly and had little or no root formation (Fig. 2).

Discussion

Factors such as protoplast plating density (1 x 10^5/ml), osmoticum (glucose) and plating technique (agarose) have already been evaluated for asparagus protoplasts by others (Kong and Chin 1988; Elmer *et al.* 1989; Kunitake and Mii 1990; Dan and Stephens 1991) and these optimal parameters were incorporated into our study. However, the effect of genotype and auxin had not been examined and showed in our study to strongly influence protoplast culture and plant regeneration.

Protoplast yields from the cell suspensions with the exception of JG 8 NAA were fairly low, particularly those from the 2,4-D cultures. The isolation procedure was the probable cause of the low yields because most of the starch filled

Fig. 2. Phenotypic differences in 2,4-D- and NAA-derived protoclones of MD10. The rooting response and shoot development from the 2,4-D-derived plants (left) was poor compared to the vigorous root and shoot growth from the NAA-derived regenerants (right).

protoplasts pelleted instead of floating. Poor recovery of protoplasts from embryogenic cells due to sucrose floatation was similarly observed in *Brassica* and *Musa* cultures (Millam *et al.* 1991; Panis *et al.* 1993). Both studies were able to increase yield by sieving only and eliminating floating, and in subsequent experiments, we found that sieving isolations alone through 61 µm and 35 µm mesh routinely yielded 4 - 6 x 10^6 protoplasts per gram of cells compared to about 1 x 10^6/gram after floatation.

Protoplasts of all four genotypes exhibited somatic embryogenesis although the degree of response was dependent on the genotype, donor cell PGR, and the presence or absence of the same PGR in the protoplast culture medium. In the study by Elmer *et al.*(1989) only protoplasts of JG 8 sustained divisions whereas protoplasts from three other genotypes, different from those used herein, failed to divide. In that study, 2,4-D was optimal for both donor callus and protoplast culture. J.G. 8 protoplasts cultured herein in 2,4-D achieved comparable PEs although frequency of plant regeneration via somatic embryogenesis was lower than previously reported via organogenesis (Elmer *et al.* 1989).

The two PGR regimes tested in this study were based on previous asparagus protoplast and somatic embryogenesis studies. 2,4-D at 5 µM has been commonly used to induce somatic embryogenesis (Levi and Sink 1991; Saito *et al.* 1991)

and was optimal for protoplast culture in a previous report from our laboratory (Elmer *et al.* 1989). NAA also induced embryogenesis and 54 µM NAA produced a higher frequency of normal somatic embryos from callus cultures that subsequently converted into plants compared to embryos induced with 5 µM 2,4-D (Levi and Sink 1991). In addition, NAA was more effective than 2,4-D in inducing divisions in protoplasts of cv. Mary Washington (Kunitake and Mii 1990). Five µM 2,4-D and 50 µM NAA were selected because both are effective in establishing embryogenic cell suspensions of asparagus (Saito *et al.* 1991; Levi and Sink 1992).

Embryo conversion was highest for Rutgers 22 from NAA cultures, from both 2,4-D and NAA for MD10, and exclusively from 2,4-D derived protoplasts from JG 8 and 86SOM1. JG 8 and 86SOM1 are both selections from the seed variety Jersey Giant, and although regeneration frequencies were similar for both, PE and colony formation differed significantly illustrating the heterogeneity of even closely related genotypes.

Treatments in which media lacking PGRs were included to potentially expedite somatic embryogenic development. Our hypothesis being that since the release of embryogenic potential typically occurs after auxin is removed from embryogenically determined cells, protoplasts derived from an embryogenic cell culture may respond similarly. This was the case since embryos developed directly from protoplasts in a rapid manner and without an intermediate callus stage.

Somatic embryogenesis from asparagus protoplasts has been previously reported to occurred after an initial callus stage and plantlet formation required 3 - 4 months (Hsu *et al.* 1990; Kunitake and Mii 1990).

Direct somatic embryo formation from protoplasts has been observed in a number of species using embryogenic calli or suspension cultures as the protoplast donor sources (Roest and Gilissen 1989; 1993). For these species, isolated protoplasts are able to maintain their cell polarity and restore it rapidly upon culture (Roest and Gilissen 1993). This response was observed previously in asparagus protoplasts although embryogenesis through a callus phase was emphasized since only a few direct embryos formed in comparison to cell colonies (Kunitake and Mii 1990).

NAA appeared to potentiate cells more than 2,4-D to give rise to embryogenically determined protoplasts capable of developing directly into somatic embryos particularly in PGR-free medium. Conversely, 2,4-D-derived protoplasts responded poorly in (-)PGR medium and the inclusion of 2,4-D was required for satisfactory divisions and for enhanced embryo formation. Similarly, protoplasts from 2,4-D derived embryogenic suspensions of *Panicum maximum* also produced relatively few divisions and exhibited no embryo formation in medium (-) PGR compared to those cultured in 2,4-D which promoted vigorous cell divisions and globular somatic embryos (Lu *et al.* 1981).

We compared embryo conversion from the 5-month-old suspension cells and from their derived protoplasts to determine if the protoplast culture scheme had an effect on plant regeneration. As well, to determine the regeneration ability of the donor suspensions which had been advised prior to protoplast isolation (Ahmed and Sagi 1993). Seven of the eight cell suspensions were capable of plant regeneration and their derived protoplasts either maintained totipotency or exhibited a significant reduction. The JG 8 NAA protoplasts rapidly divided into colonies similar to those from which they were isolated but plant regeneration did not occur. Unlike other cultures, the JG 8 NAA suspension cells lacked starch which has been related to low regeneration potential (Hirosawa, 1992).

The majority of cytogenetic variability was observed in 2,4-D derived protoclones; although, a direct comparison of the effects of 2,4-D and NAA on such variability cannot be made since equal PGR concentrations were not used. However, our observations on somaclonal variation should be taken into consideration when using the same levels of PGRs and culture protocol as listed in this study. There are reports of plants with normal karyotypes regenerated from asparagus protoplasts when 2,4-D was either in the donor culture medium (Kunitake and Mii 1990) or in the protoplast culture medium (Kong and Chin 1988). However, Elmer *et al.* (1989) regenerated only aneuploid JG 8 plants when donor callus and protoplasts were

cultured in 2,4-D. Our results corroborate with Elmer *et al.* (1989) since only aneuploid J.G. 8 plants were recovered when 2,4-D was used exclusively at the same level.

Two observations indicated that cytogenetic variability probably existed in the donor cells. Firstly, two plants regenerated from 2,4-D derived Rutgers 22 protoplasts cultured in the absence of 2,4-D had the same amount of variation as from the same protoplasts cultured in the presence of 2,4-D. Plants regenerated from MD10 suspensions were phenotypically identical to those derived from protoplasts; NAA-derived plants were vigorous and rooted well and those from the 2,4-D suspension were stunted and rooted poorly similar to their protoplast counterparts. Fitter and Krikorian (1988) found considerable somaclonal variation among *Hemerocallis* plants regenerated from embryogenic suspensions and their derived protoplasts compared with organogenic cultures. They concluded that the majority of the variation arising in the suspension and protoplast-derived plants originated from the cytogenetically heterogeneous donor cells.

All four asparagus genotypes were capable of plant regeneration from protoplasts through our system. Potentially, other genotypes could also be regenerated if a source of embryogenic donor cells can be established. Developing a protoplast culture system for a given genotype that favors direct somatic embryogenesis may aid direct-gene-

transfer and somatic hybridization studies by hastening plant regeneration and decreasing the time in culture.

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Chapter Three

Agrobacterium-mediated transformation of embryogenic suspension cells of *Asparagus officinalis* L.

ABSTRACT

Asparagus officinalis L. has been transformed with *Agrobacterium tumefaciens* in previous studies although the frequency at which transgenic tissues and plants have been produced was very low. In an effort to develop an efficient *Agrobacterium* transformation protocol, highly regenerable embryogenic suspension cultures were used as the target cells and important transformation parameters were evaluated via transient GUS expression. The binary vector, pCNL56, possesses an intron containing GUS gene that can only be expressed in plant cells which was used in all transient expression experiments. Cocultivation duration was optimal at 4 days and GUS expression was greatest at an inoculum density of 5 x 10^7 cfu/ml. Four *A. tumefaciens* strains, EHA105, GV3101(pMP90), GV3101(pGV2260) and LBA4404, were tested and EHA105 and GV3101(pMP90)produced the greatest number of GUS foci. GV3101(pGV2260) gave significantly few GUS foci but LBA4404 was unresponsive. In addition, GUS expression with EHA105 and GV3101(pMP90) induced with acetosyringone was significantly greater than with uninduced bacteria. Kanamycin, G418 and glufosinate-ammonium were evaluated as potential selective agents for stable transformation studies.

Glufosinate was effective from 2 - 5 mg/l, G418 inhibited growth at 100 mg/l and kanamycin was not inhibitory at 100 mg/l. The optimal parameters from the transient expression experiments were combined with EHA105 containing pGPTV-BAR and transgenic tissues were selected on 2 mg/l glufosinate. A single transgenic plant was produced in which integration of the BAR gene was confirmed via Southern hybridization. Transient and stable GUS expression were compared in the same experiment with both EHA105 and GV3101(pMP90). Transient expression was ten fold greater than stable expression as measured by solid blue colonies or somatic embryos after 8 weeks of culture on 100 mg/l G418. This indicates that the T-DNA is entering the cells but integration into genomic DNA is low which is the most probable reason for the low frequency of stably transformation tissues.

Introduction

Genetic transformation has the potential to increase the productivity of horticultural crops as well as to improve individual desired traits. *Asparagus officinalis* L. is one such crop that would benefit from this technology. For example, herbicide resistance would be useful for asparagus seed production in addition to enhancing cultural practices. To produce a transgenic hybrid asparagus cultivar, at least one of the parents (male or female) must be transformed. Since inbreeding would destroy the genetic integrity of the transgenic parent, it must remain hemizygous for the transgene. Assuming that the introduced gene was present at a single locus in the transgenic parent, the resulting hybrid population would segregate 1:1 for the new trait. However, a seedling population could be treated with the herbicide for which the transgene confers resistance and thus eliminate the non-transgenic plants (Conner and Abernethy 1996). The BAR or PAT genes, or modified EPSPS gene that confers resistance to the non-selective herbicides L-phosphinothricin (Liberty), and glyphosate (Roundup), respectively, would work very well for this purpose.

Asparagus officinalis L. is susceptible to certain diseases and pests that could be overcome through transformation. Asparagus can harbor several viruses (Mink and Uyeda 1977; Falloon *et al.* 1986). Among these, asparagus virus I (AV-I), a potyvirus, and asparagus virus II (AV-II),

an ilarvirus, have been reported in the United States, Europe and the Orient. The presence of either or both of these viruses is associated with a decline in field vigor and productivity, a decrease in rooting capacity and survival in tissue culture, and an increase in susceptibility to Fusarium crown and root rot (Yang 1979; Evans and Stephens 1989a; 1989b). AV-II is the most prevalent virus in Michigan asparagus fields (Hartung *et al.* 1985; Evans *et al.* 1990), although AV-I also occurs at a lower frequency (Evans and Stephens 1989a; Evans *et al.* 1990). Transforming the coat protein cDNA gene of the virus of interest into a susceptible plant has conferred coat protein-mediated protection of that virus in a number of crops (Gonsalves and Slightom 1993). The AV-II coat protein gene has been cloned and could be used to produce transgenic asparagus resistant to the AV-II virus (D. Plunkett 1995, pers. comm.).

The common and spotted asparagus beetles are serious insect pests of asparagus (Putnam *et al.* 1983). The common asparagus beetle overwinters as an adult, emerges in April and begins feeding and laying eggs on the emerging spears. Consequently, spears that are covered with eggs or have feeding damage may be rejected by the broker or processor. The spotted asparagus beetle emerges later and primarily feeds on the fern reducing the vigor of the plant. The larvae feed on the fern as well as ripening berries causing additional damage. *Bacillus thuringiensis* var. *tenebrionis* produces the

CryIII δ-endotoxin that is insecticidal to coleoptera (beetles) and harmless to humans (Estruch *et al.* 1997). The *cryIIIA* gene has been cloned and transformed into potato plants where it provided protection against the Colorado potato beetle (Perlak *et al.* 1993). If the CryIII δ-endotoxin is insecticidal to asparagus beetles, transformed asparagus containing the *cryIIIA* gene would provide control for these pests and reduce insecticide input to the crop.

"Tip breakdown" is a serious postharvest disorder of asparagus in which freshly harvested asparagus spears deteriorate rapidly and become unsaleable within 2-6 days at ambient temperatures (Lipton 1990). The spear tips show a rapid decline in respiration rate, lose protein and soluble carbohydrates, and accumulate ammonium ions (King *et al.* 1990; Lill *et al.* 1990). Regulating the gene(s) that are responsible for ammonium accumulation or assimilation (glutamine dehydrogenase, glutamine synthase) might not be a worthwhile approach since the spear tip already has an efficient system that reassimilates large amounts of potentially toxic ammonium (Hurst and Clark 1993; Downs *et al.* 1996). A better approach to overcoming tip breakdown would be to prevent proteolysis following harvest either by providing more carbohydrate (starch or sugars) for use as respiratory substrate or by slowing its present rate of usage (Hurst and Clark 1993). Altering gene expression in the sucrose synthase

pathway may be the most likely approach to delay tip breakdown (Irving and Hurst 1993).

To be able to produce transgenic asparagus with these improved traits, an efficient transformation system must be developed. Asparagus is susceptible to infection by *Agrobacterium tumefaciens* and transgenic asparagus plants have been produced via *Agrobacterium*-mediated transformation (Bytebier *et al.* 1987; Conner *et al.* 1988; Delbreil *et al.* 1993). However, only one to few transgenic plants were produced in each report indicating the low efficiency of the transformation systems. There has been one report of direct-gene-mediated transformation of asparagus protoplasts (Mukhopadhyay and Desjardins, 1994). Kanamycin resistant calluses were produced that were also positive for GUS expression, although no transgenic plants were regenerated.

The approach used herein to increase the efficiency of asparagus transformation was to target somatic embryogenic cells from suspension cultures with *A. tumefaciens* and monitor transient tranformation events with an intron containing GUS gene. It is generally considered that somatic embryos are derived from single cells (Haccius 1978). Therefore, targeting such cells from highly regenerable embryogenic cultures is an efficient pathway for producing non-chimeric, genetically transformed plants (Litz and Gray 1995). As previously stated, *Agrobacterium*-mediated transformation of asparagus has yielded limited amounts of transgenic plants.

However, *Agrobacterium* does have certain advantages over direct-gene-mediated transformation, such as higher rates of transformation, and more efficient and predictable patterns of gene integration, that warrant further development of an efficient *Agrobacterium* system (Smith and Hood 1995). The effectiveness of various transformation parameters was monitored by transient GUS expression. To prevent false positives from GUS expression in *Agrobacterium*, an intron-containing GUS gene was used that is only expressed in plant cells due to the presence of a modified plant intron in the GUS gene coding region (Li *et al.* 1992; Ritchie *et al.* 1993). To date, kanamycin is the only selective agent that has been used to select for transgenic asparagus tissues (Bytebier *et al.* 1987; Conner *et al.* 1988; Delbreil *et al.* 1993). However, monocots are relatively insensitive to kanamycin and using a more effective selective agent may benefit the production and selection of transgenic tissues (Wilmink and Dons 1993).

In this study, I attempted to increase the *Agrobacterium* transformation frequency of asparagus by employing highly regenerable embryogenic suspensions as the target cells, evaluating several selective agents for efficient selection, and using an intron-containing GUS gene to monitor transient transformation expression of each parameter tested.

Materials and Methods

Plant material

Asparagus officinalis L. cv. Rutgers 22 was micro-propagated according to Slimmon *et al.*(1985). The genotype Rutgers 22 was used because it is embryogenic as determined from the previous protoplast study.

Establishment of embryogenic cell suspensions

Elongated shoots taken from micropropagated crowns of Rutgers 22 were cut transversely into 1 - 2 cm explants, each possessing lateral buds, and placed on Linsmaier and Skoog (LS) medium (1965), containing 2% sucrose, 0.8% Bacto agar (pH 5.8) and 50 µM NAA. Cultures were incubated at 26°C, and 25 $\mu molm^{-2}s^{-1}$ light supplied by Philips F96T12/CW cool white bulbs on a 16-h photoperiod. Callus originating from the lateral buds was subcultured monthly on the same medium. After three subcultures, yellowish-white embryogenic callus possessing immature somatic embryos that formed at the surface of mucilaginous callus were used to initiate cell suspensions.

Approximately 250 mg of callus was inoculated into each of four 250 ml Erlenmeyer flasks each containing 40 ml of liquid medium of the same composition as the callus medium. The cultures were incubated at 26°C, under 4 $\mu molm^{-2}s^{-1}$ illumination for 16-h on a gyratory shaker at 110 rpm. Subcultures were performed weekly by sieving cells through 1 mm nylon mesh and transferring approx. 0.5 ml of cells,

settled cell volume (SCV), to 40 ml fresh medium. The suspensions were maintained on a pedigree basis for the first month and then one of the four cultures was visually selected for further subculture based on the presence of pro-embryogenic masses (PEMs) and clusters of globular somatic embryos. New cultures were initiated every three months.

Agrobacterium strains

The non-oncogenic *A. tumefaciens* strains used in this study are listed in Table 1. EHA105 was kindly provided by S.

Table 1. Disarmed *Agrobacterium tumefaciens* strains and their relevant characteristics.

Strain	*vir* plasmid	Antibiotic resistance	Characteristics	Reference
EHA105	pEHA105	Rif	C58 chromosome; agropine type	Hood *et al.* 1993
GV3101	pMP90	Rif, Gm	C58 chromosome; nopaline type	Koncz and Schell 1986
GV3101	pBV2260	Rif, Cb	C58 chromosome; octopine type	Deblaere *et al.* 1985
LBA4404	pAL4404	Rif	Ach5 chromosome; octopine type	Hoekema *et al.* 1983

Rif, rifampicin; Gm, gentamycin; Cb, carbenicillin.

Gelvin (Purdue Univ., USA), GV3101(pMP90) from C. Koncz (Max-Planck Institute, Germany), and GV3101(pGV2260) from J. Cardoen (Plant Genetic Systems, Gent, Belgium).

Bacteria culture

The following protocol was used for those experiments in which the *Agrobacterium* virulence genes were induced prior to transformation (induction protocol). Bacteria from -80°C freezer stocks were streaked onto 100 x 15 mm Petri dishes containing semi-solid AB medium(Chilton *et al.* 1974) with the appropriate antibiotics and incubated for three days at 28°C in darkness. A colony was placed in a 16 x 125 mm tube containing 2.5 ml of YEP medium (Chilton *et al.* 1974) with antibiotics and was grown overnight at 28°C and 250 rpm. The next morning 1.0 ml of the overnight culture was added to 25 ml of AB medium with antibiotics in a 125 ml flask and incubated at 28°C and 200 rpm (7 - 9 hr) until an optical density at 600 nm (OD_{600}) of 0.8 - 1.0 was reached. The cultures were diluted to OD_{600} = 0.3 in 25 ml induction medium (IM) that consisted of AB salts, 2 mM sodium phosphate buffer, pH 5.6, 20 mM MES, 0.5% glucose and 100 µM acetosyringone (AS) (Li *et al.* 1992). The bacteria culture was incubated overnight at 25°C and 200 rpm until the OD_{600} reached 0.8 - 1.2. The induced bacteria were then centrifuged for 10 min at 20°C and 4000 × *g*, and resuspended at a particular density in 20 ml of IM lacking antibiotics.

Non-induced bacteria was prepared by the following protocol (non-induced protocol). A fresh bacteria colony was inoculated into 2.5 ml of YEP medium with antibiotics in a 150 x 16 mm tube and incubated overnight at 250 rpm and 28°C. The

next morning, 2.0 ml of bacteria was placed into 25 ml of AB medium with antibiotics in a 125 ml flask. The culture was incubated at 200 rpm and 28°C until the OD_{600} reached 0.8 - 1.0. After reaching the proper density, the culture was centrifuged for 10 min at 4000 × *g* and resuspended to the proper density AB medium without antibiotics. For growth and maintenance of *A. tumefaciens*, the antibiotics rifampicin, carbenicillin, gentamycin, and kanamycin were used in the media at concentrations of 10, 25, 50, and 100 µg/ml, respectively.

Binary vectors

The binary vector used for all GUS expression studies was pCNL56 (Li *et al.* 1992), kindly provided by S. Gelvin (Purdue Univ., USA). pCNL56 is a 15.4 kb pBIN19-derived vector that possesses the NPT-II gene and a GUS reporter gene between its T-DNA borders. The expression of the GUS gene is driven by the *mas*/35S promoter and the coding region contains a modified plant intron for plant cell specific expression (Raineri *et al.* 1990). All of the strains listed in Table 1 containing pCNL56 were tested for transient GUS expression in tobacco prior to asparagus transformation.

The binary vector pGPTV-BAR was used for the BAR gene transformation studies (Becker *et al.* 1992), and was kindly provided by D. Becker (Max-Planck Institute, Germany). pGPTV-BAR is a 13.4 kb pBIN19-derived vector containing the BAR gene

driven by the *nos* promoter. The BAR gene in this vector is derived from *Streptomyces hygroscopicus,* codes for phosphinothricin acetyltransferase (PAT), and confers resistance to the non-selective herbicides glufosinate and bialaphos (White *et al.*, 1990). The integrity of the BAR gene was confirmed by transforming tobacco leaf explants with the vector and regenerating and rooting transgenic shoots on lethal levels of glufosinate ammonium (5 mg/l). The binary vectors were transformed into *A. tumefaciens* via a modified freeze-thaw method (Chen *et al.* 1994).

Histochemical GUS assay

Transient and stable GUS expression was determined histochemically according to the procedure of Jefferson (1987). Unless stated otherwise, cells from a single dish (replication) were placed into a well of a Corning 6-well dish and stained with 1.5 ml of GUS solution containing 1 mM X-gluc in 100 mM phosphate buffer, 10 mM EDTA and 0.1% Triton X-100 overnight at 37°C. Tissues were not fixed before staining. GUS expression (GUS foci) was scored as isolated blue spots on cell colonies or somatic embryos, a tight group of spots, or solid blue cell colonies or embryos. Data were analyzed by analysis of variance using MSTAT-C (version 1.2). Least significant differences (LSD) were calculated at the 5% level of probability.

Experiments

Evaluation of selective agents

Kanamycin (Sigma), G418 (Geneticin; Boehringer Mannheim) and glufosinate ammonium (Hoechst-Roussel) were added to embryo maturation (EM) medium composed of MS salts, 0.4 mg/l thiamine, 100 mg/l inositol, 20 g/l sucrose and 1% phytagel (Sigma) at 0, 12.5, 25, 50 and 100 mg/l. The selective agents were filter sterilized (0.22 µm) and added to warm (60°C) media after autoclaving. Five 100 x 15 mm dishes each containing 30 ml of medium were prepared for each treatment.

Embryogenic suspensions cultures were harvested 5 days after the previous subculture, sieved through 1 mm nylon mesh and washed three times with medium composed of MS salts, 0.4 mg/l thiamine, 100 mg/l inositol, and 20 g/l sucrose (pH 5.7)(EMW). 0.5 ml of washed cells (SCV) was resuspended in 50 ml of EM. One ml of the washed suspension (10 - 15 mg of cells) was placed on each dish. The treatments were arranged in a randomized complete block (RCB) design and incubated for four weeks at 26°C and 25 $\mu molm^{-2}s^{-1}$ illumination for 16-h. Data were taken on fresh weight at the end of four weeks and presented as percent of the control.

Based on the results of the above experiment, glufosinate was further tested to determine optimal levels for transformation selection. Glufosinate ammonium was added to EM medium at 0, 1, 2, 3, 4 and 5 mg/l. Five 100 x 15 mm dishes were prepared for each treatment. Cells were added to

each dish as described above. The treatments were arranged in a RCB design and incubated for four weeks at 26°C and 25 μmolm^{-2}s^{-1} illumination for 16-h. Data were taken on fresh weight at the end of four weeks and presented as percent of the control.

Cocultivation duration for optimal transient GUS expression

EHA105:pCNL56 was prepared by the non-induced protocol and adjusted to 5 x 10^8 colony forming units per milliliter (cfu/ml) in EMW. Embryogenic suspensions were sieved through 1 mm nylon mesh, washed 3X in EM and 1.5 g of washed cells was placed in each of four 100 x 15 mm dishes. The cells were inoculated with 20 ml of bacteria for 15 min on a gyratory shaker at 40 rpm and 25°C in darkness. After inoculation, the bacteria was pipetted off and 250 mg of cells was placed on each of 20 100 x 15 mm EM dishes. One and a half grams of uninoculated control cells was handled identical to the inoculated cells and 250 mg of those cells were placed on five EM dishes. The cultures were cocultivated in darkness at 25°C. Five random inoculated dishes were subcultured to EM medium with 300 mg/l timentin (EMT) at 2, 3, and 4 days and incubated under the same conditions. Timentin is a bacteriostatic antibiotic used to prevent growth of Gram negative bacteria such as *Agrobacterium*. The fourth inoculated treatment remained on EM medium for the entire 6 days. At 6 days, the cells of each inoculated dish and the

control was stained for transient GUS expression. Data were taken on the number of GUS foci per sample.

Effect of *Agrobacterium* strain and acetosyringone on transient GUS expression

The four strains listed in Table 1 containing pCNL56 were prepared for transformation by the induction protocol and by the non-induced protocol to compare the two methods. Each strain lacking pCNL56 was prepared by the non-induced protocol as the negative controls. All bacteria were adjusted to a density of 5 x 10^8 cfu/ml prior to transformation. Embryogenic suspensions were sieved through 1 mm mesh, washed three times with EMW and 250 mg of cells were placed in each of five 60 x 15 mm dishes per treatment. The cells in each dish were inoculated with 6 ml of bacteria for 15 min at 25°C and 30 rpm on a gyratory shaker. Following inoculation, the agrobacterium was pipetted off and the inoculated cells were plated onto 100 x 15 mm dishes containing solidified EM medium with 100 µM AS (EMAS). Those treatments with non-induced bacteria were placed on EM medium without AS. The inoculated negative controls for each strain were plated onto two dishes containing EM medium. All of the cultures were cocultivated for 4 days at 25°C in darkness. Subsequently, the cultures were placed on EMT medium for two additional days under the same environment. The cultures were assayed for transient GUS

expression after six days of culture and the number of blue foci was scored in each treatment.

Effect of inoculum density on transient GUS expression

Embryogenic suspensions were prepared for transformation by sieving through 1 mm^2 mesh and washed three times with EMW. Cells, 250 mg, were placed in each of six 60 x 15 mm dishes for each of the five treatments. *Agrobacterium* strain EHA105:pCNL56 was prepared for transformation by the induction protocol. Five 40 ml aliquots of the bacteria were adjusted to 10^7, 5 x 10^7, 10^8, 5 x 10^8 and 10^9 cfu/ml in IM medium prior to transformation. Six milliliters of a bacteria suspension were placed in each of the six dishes containing embryogenic material for each density treatment. The cultures were inoculated for 15 min at 25°C and 30 rpm on a gyratory shaker. Following inoculation, the bacteria was removed and the cells were placed on 100 x 15 mm dishes containing EMAS medium. The cultures were cocultivated for four days in darkness at 25°C. After four days the cells were transferred to EMT dishes and incubated under the same conditions for an additional two days. Subsequently, the cells were assayed for GUS expression and scored for the number of blue foci.

Effect of inoculation duration with EHA105:pGPTV-BAR on the recovery of putative transgenic plants from embryogenic suspension cells

A. tumefaciens strain EHA105 containing pGPTV-BAR or lacking pGPTV-BAR (control bacteria) were prepared by the induction protocol and adjusted to 5 x 10^7 cfu/ml prior to transformation. Embryogenic suspension cultures were sieved through 1 mm^2 mesh and washed three times in EMW prior to inoculation. The embryogenic cells were inoculated for 15 min, 1 hr and 8 hr at 25°C in darkness and 30 rpm on a gyratory shaker. Following inoculation, the bacteria was removed and 250 mg of cells were placed on each 100 x 15 mm dish containing EMAS medium and cocultivated for 4 days in darkness at 25°C. Subsequently, the three treatments and the negative control were transferred onto 100 x 20 mm dishes containing EMT medium with 2 mg/l glufosinate (EMTI medium). The positive control was placed on EMT medium. The dishes were arranged in a RCB design and incubated at 26°C and 25 $\mu molm^{-2}s^{-1}$ illumination on a 16-h photoperiod.

In the first experiment, the positive and negative controls were inoculated for 15 min only and two dishes of each were prepared. Three replications of each of the three treatments were also prepared. In the second experiment, two negative and two positive control dishes were prepared for each inoculation duration, and six replications were made for each of the three treatments. The cultures were subcultured

to fresh EMTI and EMT dishes every two weeks for a total of six weeks.

Following culture on EM media, all bipolar embryos from each treatment were subcultured to 100 x 20 mm dishes containing embryo germination medium (EGTI) composed of MS salts and vitamins, 100 mg/l inositol, 0.65 mg/l ancymidol, 40 g/l glucose, 300 mg/l timentin, 4 mg/l glufosinate, and 8 g/l Bacto agar (pH 5.7). Twenty five bipolar embryos from the EMT positive control dishes were placed on each of four EGTI dishes as negative germination controls and onto four EGT dishes lacking glufosinate as positive germination controls for both experiments. The cultures were subcultured to fresh medium of the same composition at three weeks and cultured for an additional three weeks. The cultures were incubated under illuminated conditions as previously described. Putative transgenics that had a visible root and shoot in comparison to the negative control embryos were grown further by being placed individually into 150 x 25 mm culture tubes containing 20 ml of EGTI medium (5 mg/l glufosinate). Germinated embryos from the positive control treatment were placed individually into 150 x 25 mm tubes containing EGT medium and EGTI medium for positive and negative controls, respectively.

Effect of *Agrobacterium* strain and inoculation duration on transient and stable GUS expression

Agrobacterium strains EHA105 and GV3101(pMP90), both possessing pCNL56, were prepared for transformation by the induction protocol. The bacteria were adjusted to 5 x 10^7 in IM medium prior to transformation. Embryogenic suspensions were sieved through 1 mm^2 mesh, washed three times with EM medium, and 1.5 g of cells were placed in each of six 100 x 15 mm dishes. Each culture was inoculated with either EHA105 or GV3101 for 15 min, 1 hr or 8 hr (six treatments) and incubated at 25°C, in darkness and 30 rpm on a gyratory shaker. Following inoculation, the bacteria was removed and 250 mg of cells were placed on each of six 100 x 15 mm dishes containing EMAS medium for each treatment. Six control dishes containing 250 mg of uninoculated suspension cells were prepared and included in the experiment. The cultures were cocultivated for 4 days in darkness at 25°C in a RCB design. Following cocultivation, three replications were subcultured to 100 x 20 mm dishes containing EMT medium for transient expression and three replications were subcultured to 100 x 20 mm dishes containing EMT medium with 100 mg/l G418 (EMTG medium). Two control samples were subcultured to EMT for negative transient expression controls, and for stable expression two samples were subcultured to EMT medium (positive control) and two samples were placed on EMTG medium (negative control). The samples for transient expression were incubated for two

additional days in darkness and then evaluated for GUS expression. The cultures for the stable expression experiment were incubated at 26°C and 25 $\mu molm^{-2}s^{-1}$ illumination on a 16-h photoperiod, and subcultured to fresh EMTG medium every two weeks for 8 weeks. Stable GUS expression was evaluated by incubating the entire contents of each dish in the GUS solution and recording the number of colonies and somatic embryos expressing GUS.

DNA isolation and Southern analysis

Total genomic DNA was isolated from fern tissue of *in vitro* control and putative transgenic asparagus plants via the CTAB (cetyltrimethylammonium bromide) extraction procedure (Doyle and Doyle 1990). The procedure was modified by using cold (-20°C) ethanol (2.5 volumes of the DNA solution) in place of isopropanol to precipitate the DNA (Lewis and Sink 1996). The DNA was dried, dissolved in 400 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.4) and incubated with RNAse for 1 hr at 37°C. The samples were quantified by fluorometer and adjusted to a final concentration of 300 ng/µl.

The RFLP probe for the BAR gene was generated by the polymerase chain reaction (PCR). A left primer 5'-CAT-GAG-CCC-AGA-ACG-ACG-CC-3' and a right primer 5'-GCA-GGC-TGA-AGT-CCA-GCT-GC-3' were used to amplify a 512 bp fragment from the BAR gene coding sequence (White *et al.* 1990) in pGPTV-BAR. The amplifications were carried out in 50 µl reaction mixtures

containing 15 ng of pGPTV-BAR template DNA, 64 ng (200 µM) of each primer, 1 mM $MgCl_2$, 200 µM each of dNTPs, and 1.25 U Taq DNA polymerase in 1X PCR buffer. The amplification profile consisted of 4 min at 94°C, and 35 cycles of 1 min at 94°C, 1 min at 60°C, and 2 min at 72°C. The reaction was run in a Perkin-Elmer Cetus 9600 PCR system. The PCR product was checked for amplification and size by electrophoresis on a 1% agarose gel for 1.5 hr at 80 V in 1X TAE buffer followed by staining in ethidium bromide. Following product confirmation, the probe DNA was diluted to 20 ng/µl in TE buffer (pH 8.0). Probe DNA (25 ng) was labeled with radioactive (^{32}P) dCTP by the random primer method using the Gibco BRL RadPrime kit.

DNA (6 µg) of an untransformed control and the two putative transgenic asparagus plants were digested with *Hin*dIII (8 U enzyme per µg DNA) and separated on a 0.9% TAE agarose gel for 7 hr at 150 V. A lane containing 2 µg of Lambda DNA digested with *Hin*dIII was included as a DNA size marker. Blotting and hybridization were performed according to Wang *et al.* (1995). Banding patterns were visualized by exposure (Amersham Hyperfilm-MP film) to membranes in a cassette with an intensifying screen at -80°C.

Results

Evaluation of selective agents

Kanamycin, G418 and glufosinate were compared to establish which selective agent would be most effective for

selecting transgenic tissues based on growth inhibition of untransformed embryogenic asparagus cells. The cultures had the greatest sensitivity to glufosinate (Figure 1). At the lowest level tested, 12.5 mg/l, growth of the cultures was only 10% of that of the untreated control after four weeks. A small amount of a nonembryogenic white granular callus developed in this treatment although most of the tissue was yellowish brown (necrotic), and no bipolar somatic embryos were observed. Higher levels of glufosinate completely inhibited cell growth. G418 allowed for growth up to 50% of the control at 50 mg/l. The treatments containing 12.5 and 25 mg/l G418 were visually similar to the control although some growth inhibition was measured. The highest level of G418, 100 mg/l, did suitably inhibit cell growth and expression of embryogenesis. Kanamycin was the least effective of the three agents tested. Growth was still 80% of the control up to 50 mg/l. However, at 25 mg/l somatic embryos began to turn white or "bleach" from the kanamycin and at 50 mg/l only bleached embryos were present. At 100 mg/l, growth was still approx. 60% of the control which is not suitable for efficient cell selection. This highest level of kanamycin did not inhibit embryo development, however the embryos present were white versus green in the control.

The effect of the selective agents on cell viability was tested using fluorescein diacetate (FDA). Cells showed no loss of viability at the highest level of kanamycin selection.

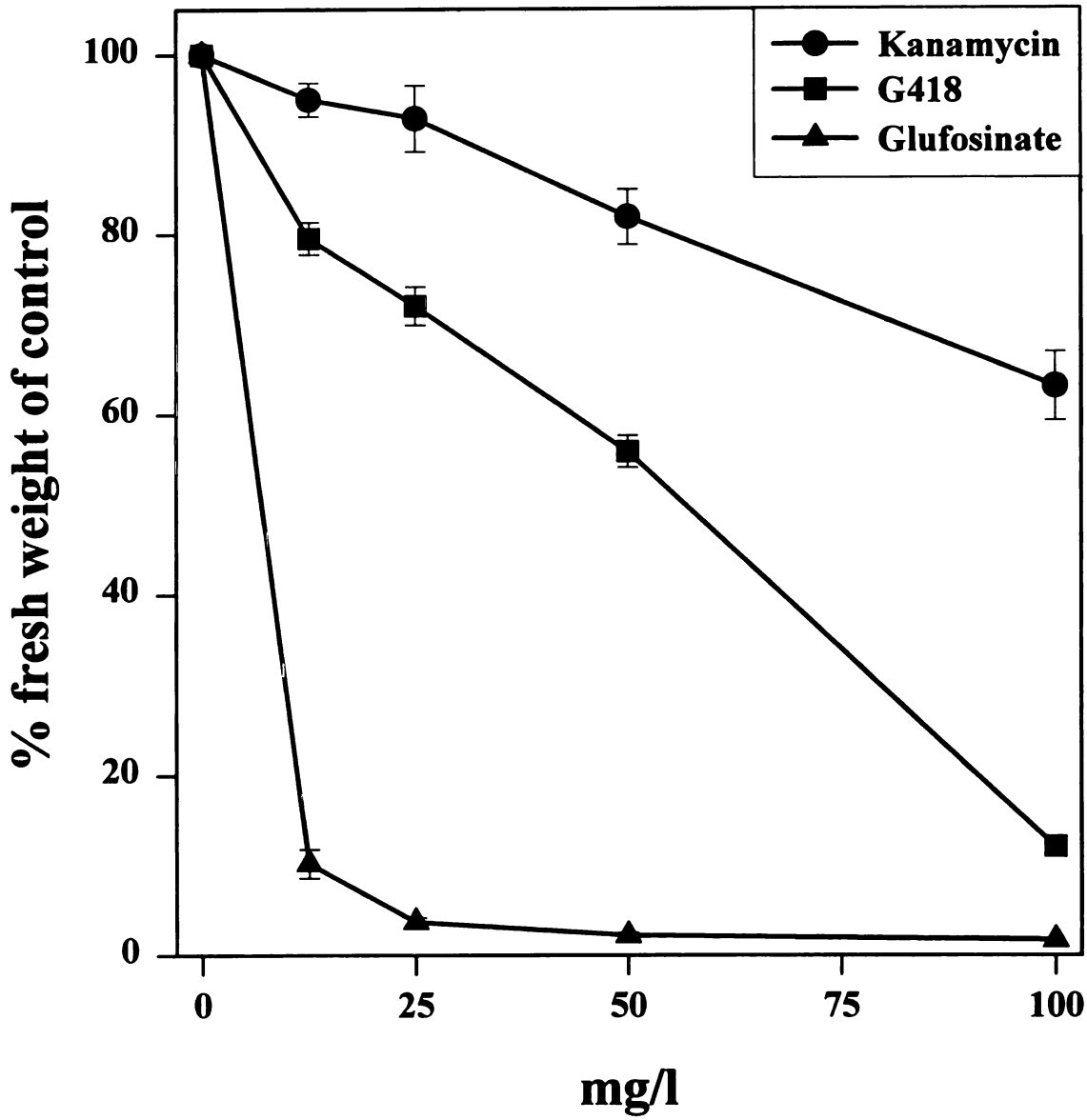

Figure 1. Comparison of the ability of three selective agents to inhibit the growth of embryogenic asparagus cells.

At 50 mg/l of G418, cell viability was approx. 50% and was less than 5% at 100 mg/l. Cells on glufosinate showed little (≤ 1%) or no viability on any of the treatments.

The selective agent experiment was repeated using only glufosinate at low levels to determine the lowest concentration to obtain suitable growth inhibition (Figure 2). At 1 mg/l, growth was significantly inhibited to 22% of the control and embryogenesis did not proceed past the globular stage although some green tissues were present. Two milligrams per liter of glufosinate inhibited growth to approx. 13% of the control. Embryogenesis was arrested and the tissues were mostly necrotic. Cultures on 3-5 mg/l glufosinate were similar with slightly less growth than occurred at 2 mg/l. Based on these data, selection for transgenic cells was performed using 2 mg/l glufosinate.

Glufosinate was also tested for its ability to inhibit the growth of germinated embryos and root and shoot growth of *in vitro* asparagus crowns. After 4 weeks on embryo germination medium with 5 mg/l glufosinate, germinated embryos (Figure 3a) and *in vitro* crowns (Figure 3b) failed to develop and quickly became necrotic. Thus, glufosinate at 5 mg/l would successfully inhibit root and shoot formation of non-transgenic escapes.

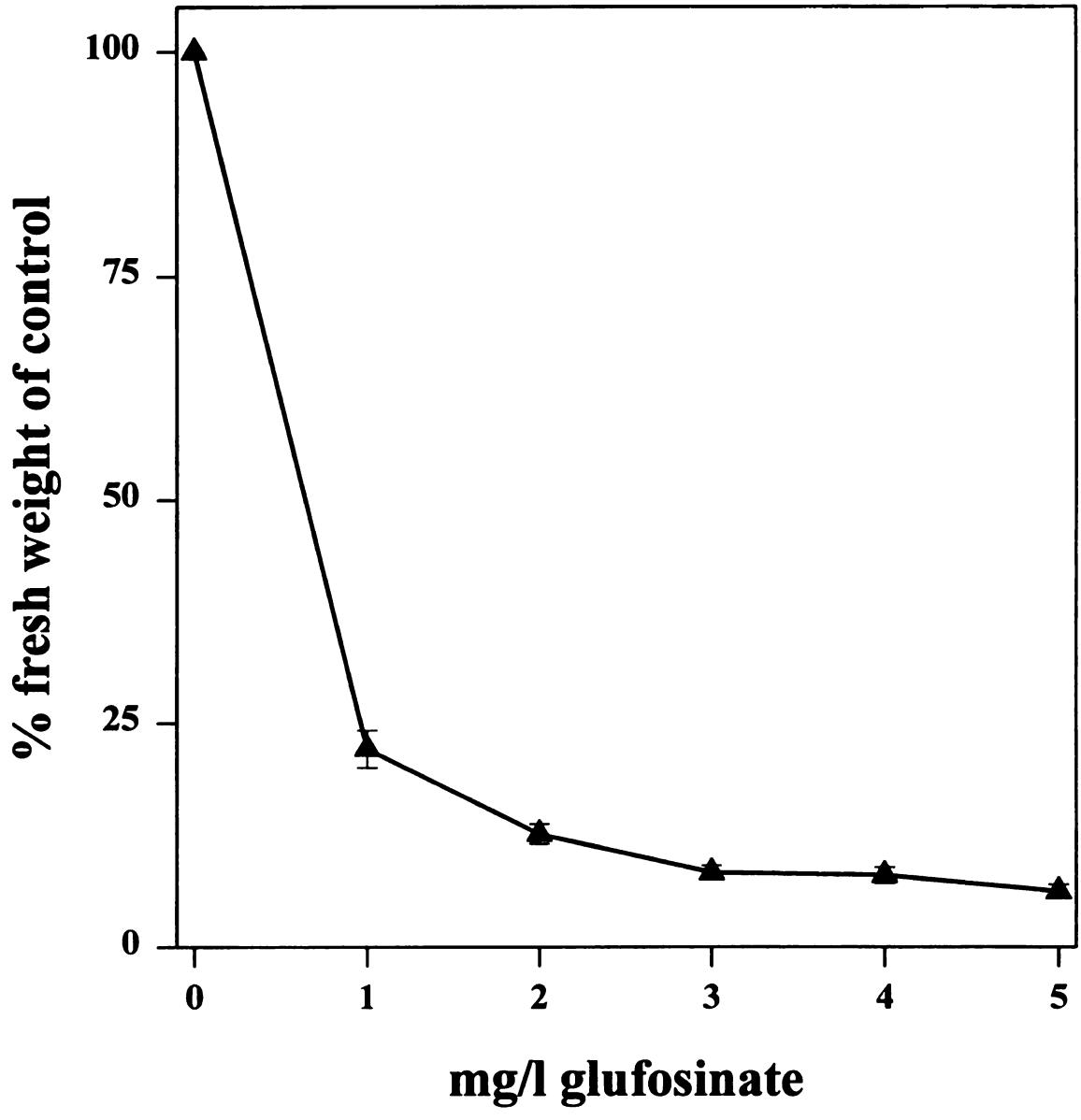

Figure 2. The effect of low levels of glufosinate on the growth of embryogenic asparagus cells.

Figure 3. Effect of 5 mg/l glufosinate on untransformed asparagus germinated embryos and *in vitro* crowns after four weeks. Glufosinate treatment on left and control on right. (A) Germinated embryos. (B) *In vitro* crowns.

Evaluation of parameters for transient GUS expression

Transient GUS expression in embryogenic suspension cells manifested itself as individual blue foci, tight groups of foci or solid blue regions ≥ 250 µm (Figure 4). These patterns of expression were consistant throughout the studies. GUS expression was never observed in the negative controls or in the *Agrobacterium* itself.

Cocultivation duration

The optimal length of cocultivation was established to maximize transient GUS expression in all future experiments. GUS expression was observed in all of the treatments and was optimal after 4 days of cocultivation (Figure 5). Four days of cocultivation produced significantly more GUS foci than 2 or 3 days. Six days produced the second greatest response and was not significantly different from 2, 3, or 4 days although the number of GUS foci was 38% less than for 4 days. Four days of cocultivation was used for all further transformation studies.

Effects of Agrobacterium strain and acetosyringone

Inducing the *Agrobacterium* prior to transformation was beneficial for transformation but only in the most responsive strains. Only the strains with the C58 chromosomal background (see Table 1) produced GUS expression in the cell cultures (Figure 6). Strain GV3101(pGV2260) gave significantly less

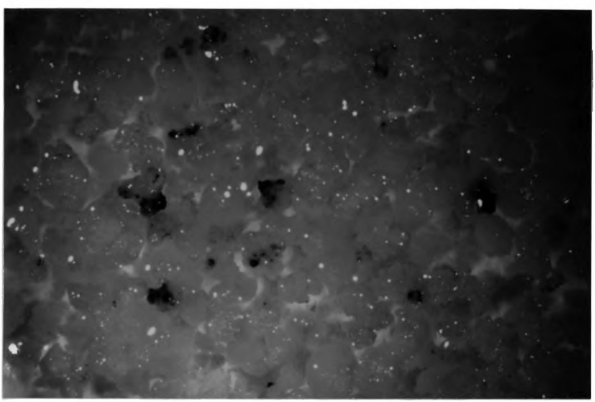

Figure 4. Typical transient GUS expression in embryogenic asparagus suspension cells.

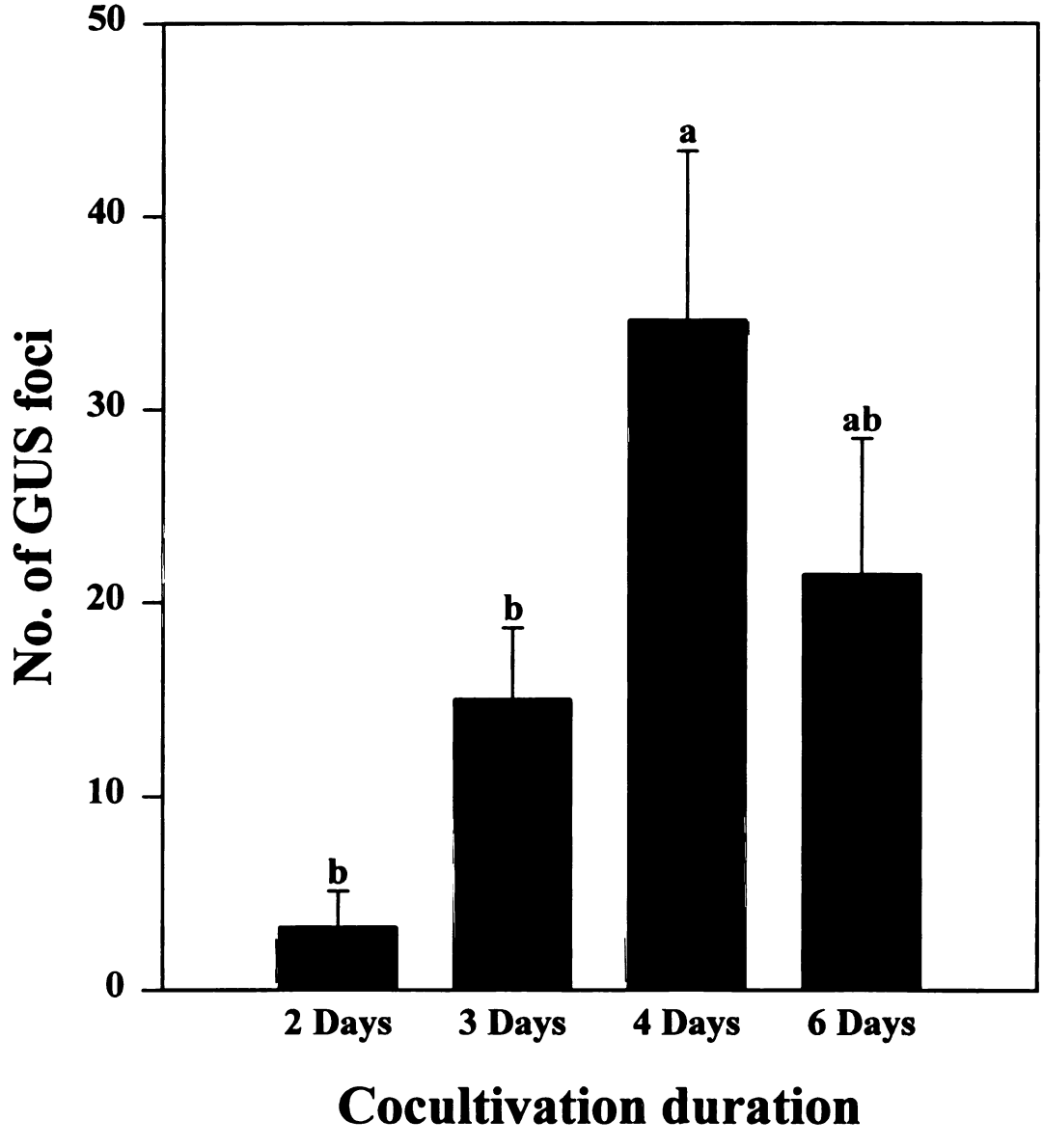

Figure 5. Effect of cocultivation duration with EHA105:pCNL56 on transient GUS expression in embryogenic asparagus cells. $LSD_{0.05}$ = 19.0.

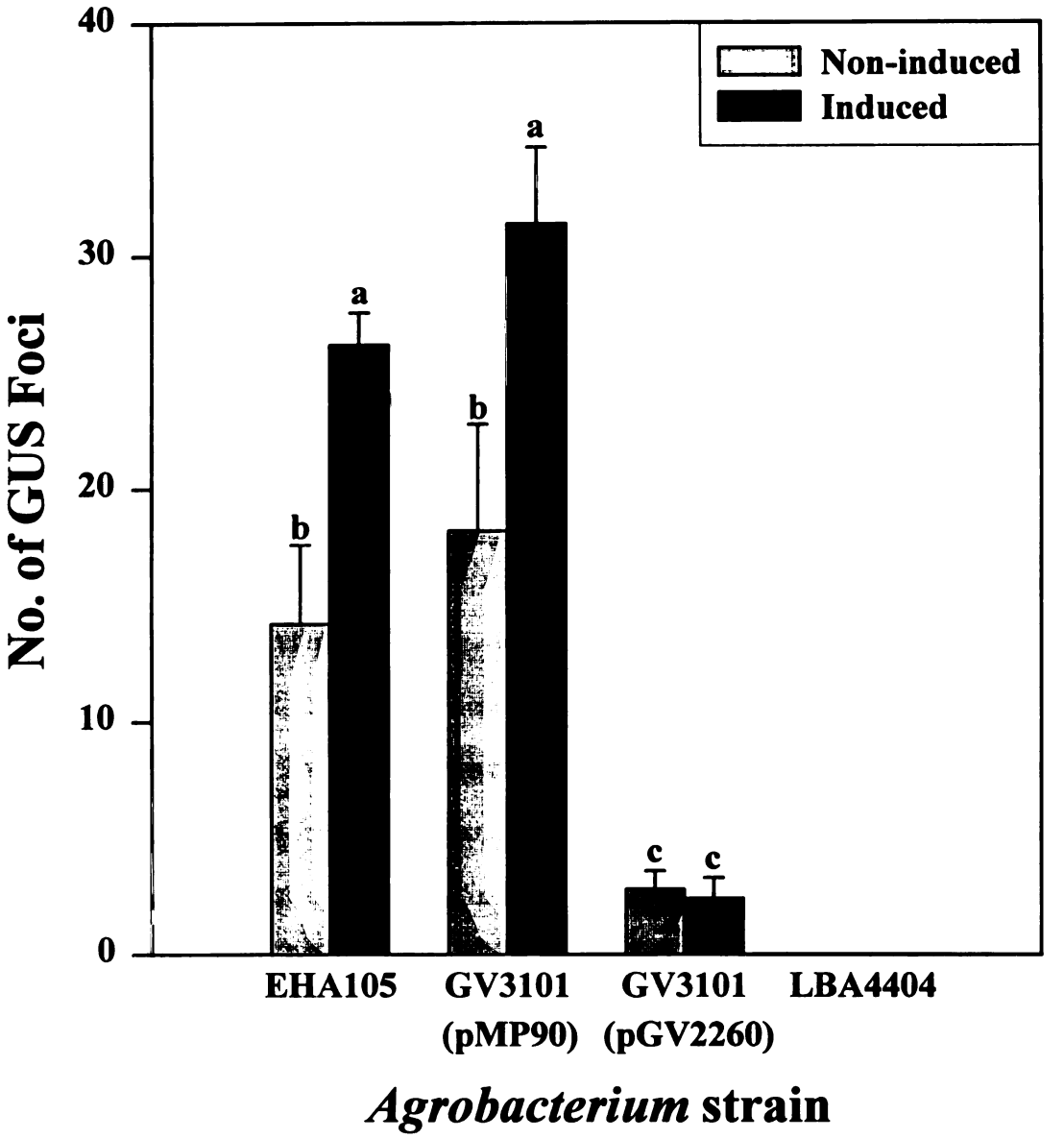

Figure 6. The effect of *Agrobacterium* strain and *vir*-gene induction on transient GUS expression in embryogenic asparagus cells. $LSD_{0.05}$ = 7.1.

expression than EHA105 and GV3101(pMP90), and induction had no effect. No expression was observed in the LBA4404 treatment. GUS expression was highest in GV3101(pMP90) treatments, although mean GUS foci were not significantly different when comparing induced cultures and uninduced cultures of EHA105 to GV3101(pMP90). GUS expression was significantly greater within those two strains when the bacteria were induced with AS.

Inoculum density

Transient GUS expression was enhanced with lower inoculum densities of EHA105:pCNL56 (Figure 7). The mean number of GUS foci was greatest at 10^7 and 5 x 10^7 cfu/ml and decreased signicantly at higher bacteria densities. GUS expression at 10^8, 5 x 10^8 and 10^9 was not significantly different and the number of foci was lowest at 10^9 cfu/ml for the entire experiment. The cells that were inoculated with bacteria at 5 x 10^8 and 10^9 took on a brownish appearance at the end of 6 days, whereas the cultures from the lower densities remained yellowish-white and healthy in appearance. Although the number of GUS foci was not significantly different between the two lowest treatments, 5 x 10^7 cfu/ml was used for all further experiments.

Stable transformation studies with EHA105:pGPTV-BAR

The optimal transformation parameters that were determined in the transient expression and the selective

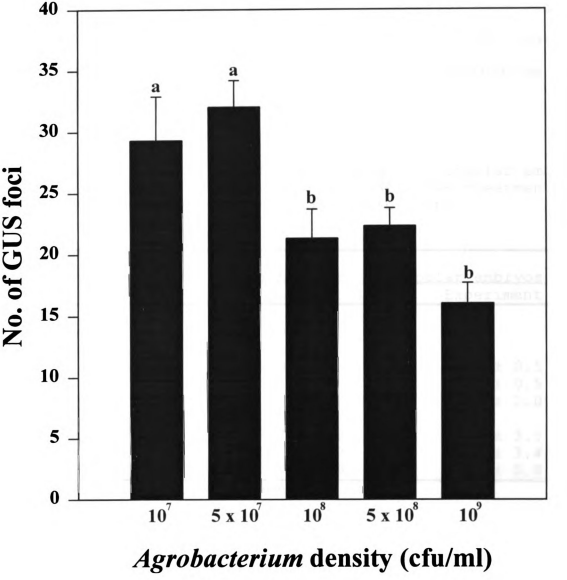

Figure 7. Effect of EHA105:pCNL56 inoculum density on transient GUS expression in embryogenic asparagus cells. $LSD_{0.05}$ = 6.6.

agents studies were applied to stable expression experiments using EHA105:pGPTV-BAR. After six weeks culture on EMTI medium considerably more green, bipolar embryos were present in the EHA105:pGPTV-BAR treatments than in the negative controls (Table 2). This was an initial indication that transgenic tissues may have been produced.

Table 2. Comparison of the number of green, bipolar embryos present in EHA105 control and EHA105:pGPTV-BAR treatments after 6 weeks of culture on 2 mg/l glufosinate.

Inoculation duration	Mean number of bipolar embryos	
	Experiment 1	Experiment 2
(+) control	> 300	> 300
15 min (-) control	1.0 ± 1	9.5 ± 0.5
1 hr (-) control		14.4 ± 0.5
8 hr (-) control		18.0 ± 1.0
15 min BAR	13.7 ± 0.9	32.3 ± 3.1
1 hr BAR	11.0 ± 3.2	31.2 ± 3.4
8 hr BAR	12.7 ± 0.9	51.7 ± 5.0

±, standard error of the mean.

Each positive control dish contained at least 300 mature embryos, whereas the negative controls contained 1 - 18 embryos on average depending on the experiment. This indicated that selection was working, although not completely, but enough to suppress the development of most non-transgenic tissues.

After six weeks of culture on EGT germination medium, approx. 95% of the positive control embryos had germinated

with elongated roots, and 26% and 53% had shoots that were ≥ 1 cm in length in the first and second experiments, respectively. In contrast, development was almost completely arrested in the negative controls. Glufosinate at 4 mg/l (EGTI medium) inhibited radical elongation in embryos past 0.5 cm, completely prevented shoot formation and caused 80 - 90% of the embryos to become necrotic. In the same time, one embryo from the 1 hr inoculation duration treatment from both experiments (2 embryos total) germinated with both roots and shoots. These two embryos did not root as vigorously as the positive controls, but they did germinate and survive selection on 4 mg/l glufosinate. These two putative transgenic plantlets were placed in culture tubes containing EGTI (5 mg/l glufosinate) for further selection and crown formation. After four weeks culture, the two putative transgenic plants survived and elongated roots on the EGTI medium (Figure 8). The untranformed control plants were completely necrotic and did not develop past the initial explant stage on the same medium. During the first four weeks of culture, the putative transgenics rooted similar to the positive controls although shoot formation was depressed. Upon subculture to the same medium and an additional four weeks of incubation for crown development, the putative transgenic from the first experiment produced a single new shoot of 3 cm and a few roots that elongated less than 2 cm. The putative transgenic from the second experiment was more

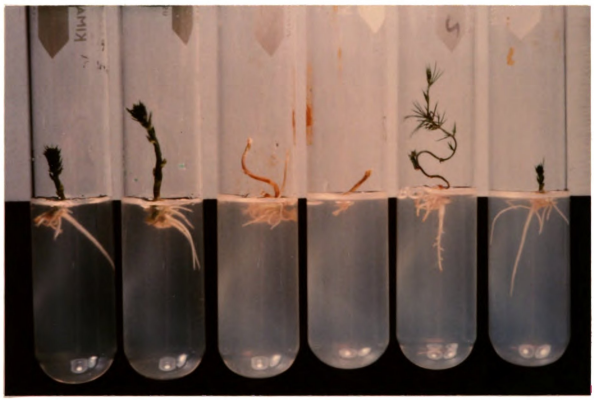

Figure 8. Appearance of putative transgenic somatic embryo-derived plants after four weeks of culture on 5 mg/l glufosinate. Left two tubes, untransformed controls without glufosinate. Middle two tubes, untransformed controls on glufosinate. Right two tubes, putative transgenics from Exp. 1 and Exp. 2, respectively, on glufosinate.

vigorous and had a root system similar to some of the positive controls and produced two new shoots up to 8 cm in length. In Figure 9, a single ~5 kb band representing the BAR transgene was resolved by Southern hybridization for the putative transgenic genomic DNA from Exp. 2 (lane 4). Bands were absent in the negative control (lane 2) and in the putative transgenic DNA from Exp. 1 (lane 3). This confirmed that only the plant from the second experiment was transgenic. Further studies could not be performed because the transformed plant became contaminated and did not survive transfer to the greenhouse.

Relating transient to stable GUS expression

Since stable transformation was very low in the previous study, transient and stable expression were compared within the same experiment to see how well the two related with the *Agrobacterium* strains used and the asparagus cell cultures. Transient GUS expression was greatest with both EHA105 and GV3101(pMP90) with either a 15 min or 1 hr inoculation time (Figure 10). Both strains were very similar in response. The mean number of GUS foci produced for those treatments was notably high for this study. Transient expression was significantly less at 8 hr for both of the strains. The 8 hr treatments did have a brownish appearance which may have been due to the detrimental effects of the bacteria and lead to reduced GUS expression.

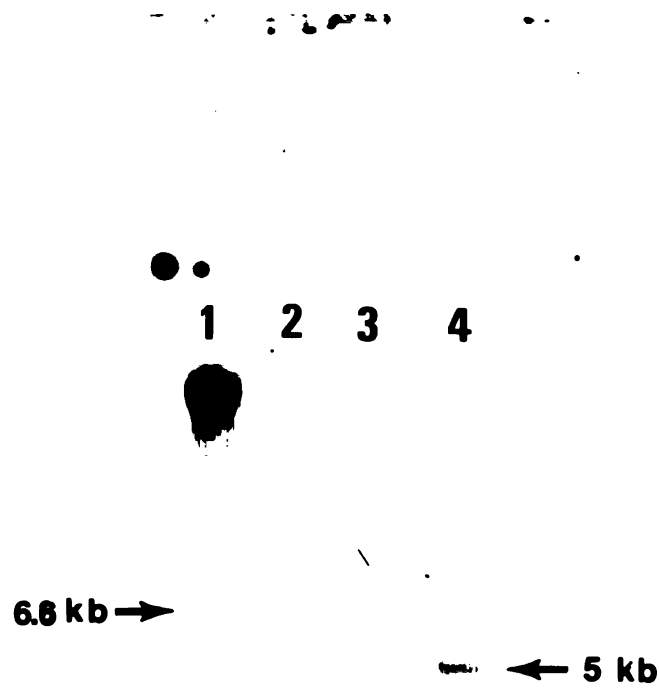

Figure 9. Southern blot for the BAR gene in putative transgenic asparagus plants transformed with EHA105:pGPTV-BAR. Lane 1, lambda DNA digested with *Hin*dIII. Lane 2, untransformed asparagus control. Lane 3, putative transgenic from Exp. 1. Lane 4, transgenic asparagus from Exp. 2 with a single T-DNA insert.

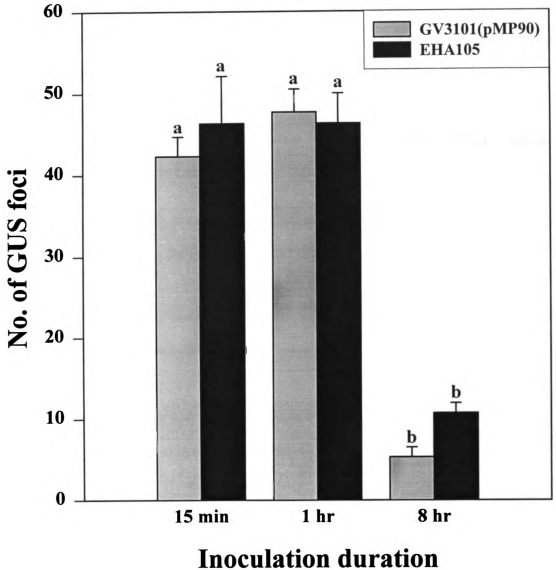

Figure 10. The effect of inoculation duration and *Agrobacterium* strain on transient GUS expression in embryogenic asparagus cells. $LSD_{0.05}$ = 11.2.

After 8 weeks of culture on 100 mg/l G418, the cultures were evaluated for stable GUS expression. Growth was suitably inhibited with 100 mg/l G418 in the negative control and no GUS expression was observed in this treatment. GUS expressing colonies and embryos (Figure 11) were produced in all of the treatments, but stable expression was ten times lower than transient expression (Figure 12). The data were not significant as determined by ANOVA.

Figure 11. Somatic embryos exhibiting stable GUS expression after 8 weeks of culture on 100 mg/l G418. Far left, uninoculated control embryo.

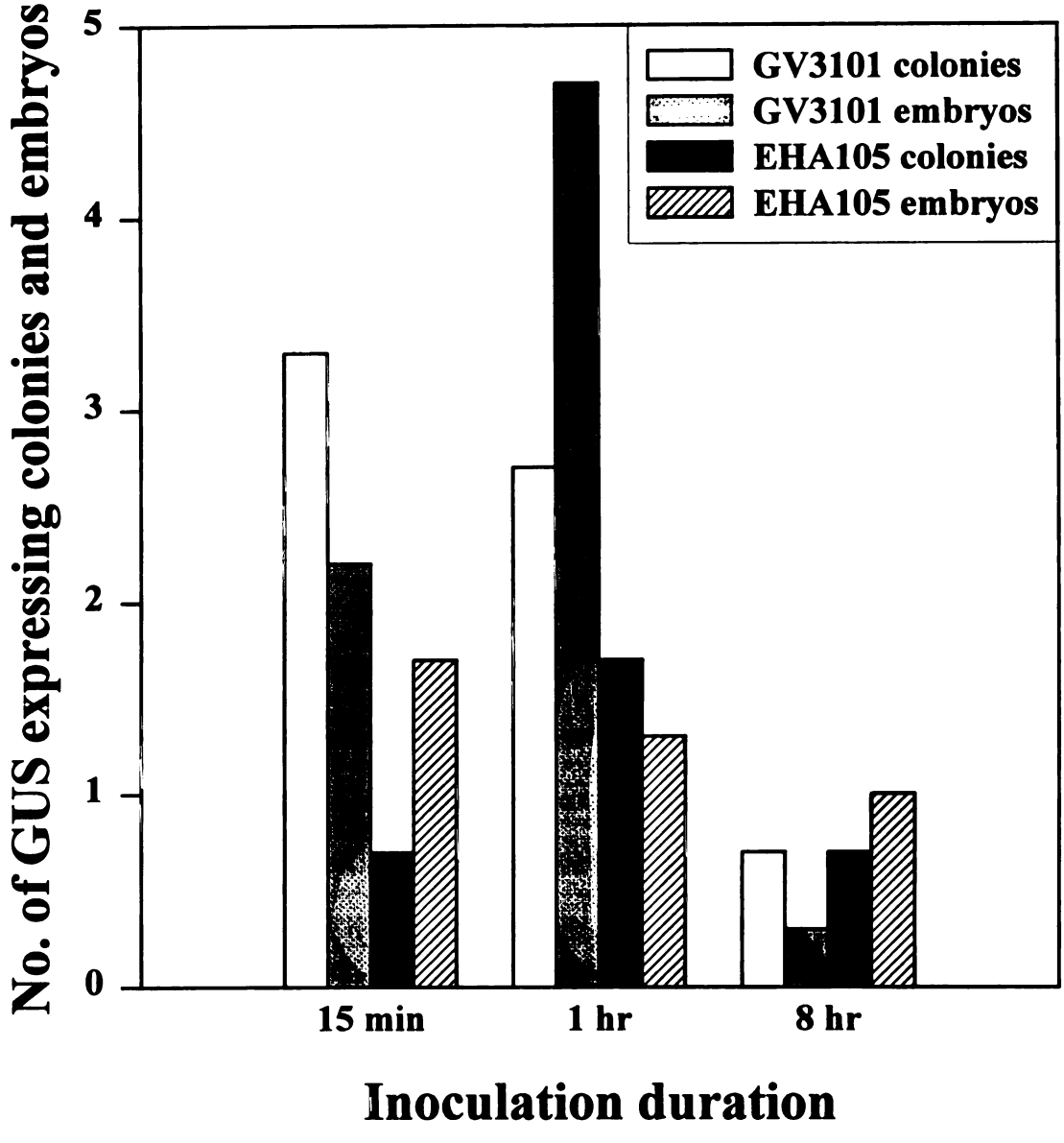

Figure 12. The effect of inoculation duration and *Agrobacterium* strain on stable GUS expression in cell colonies and somatic embryos after 8 weeks of culture on 100 mg/l G418. Treatment effects were not significant.

Discussion

An efficient *Agrobacterium*-mediated transformation system for asparagus was sought by combining highly regenerable embryogenic asparagus cell cultures with an appropriate *A. tumefaciens* strain and optimal transformation parameters. Transient GUS expression assays are ideal for optimizing transformation parameters because they provide data quickly and easily without having to select for transgenic tissues. Such assays have been found useful for optimizing parameters that subsequently are applied to the production of transgenic plants (Janssen and Gardner 1989; Van Wordragen *et al.* 1992; Janssen and Gardner 1993).

However, in this study transient expression assays were not found to be good predictors for optimizing stable transformation. It should be noted that transient expression is not an indication of integration but rather an assessment of T-DNA transfer and transcription within the plant cells. *A. tumefaciens* strain EHA105 was used in several preliminary studies and was continued to be used because it produced consistant transient GUS expression. In contrast, when the optimum parameters derived from the transient expression studies were applied to stable expression using EHA105:pGPTV-BAR, only one transgenic plant was recovered. This finding prompted further investigations into the relationship between transient and stable expression relative to asparagus. The wildtype strain A281 is the progenitor to EHA105 and failed to

produce swellings or galls on spears of 17 *A. officinalis* genotypes (Conner *et al.* 1988). Although lack of tumorigenesis does not necessarily mean the absence of stable transformation especially in monocots (Godwin *et al.* 1992), there is a strong possibility that the frequency of T-DNA integration is quite low using EHA105 in asparagus. *A. tumefaciens* strain GV3101(pMP90) was included along with EHA105 because it had been reported to produce transgenic asparagus tissues in other studies (Hernalsteens *et al.* 1984; Conner *et al.* 1988; Prinsen *et al.* 1990; Delbreil *et al.* 1993). Transient GUS expression in the final study had occurred at levels exceeding previous experiments for both strains, but after 8 weeks on selection medium(100 mg/l G418), the number of colonies and somatic embryos expressing GUS was ten-fold lower than for the initial transient expression assay at 6 days. Apparently, the T-DNA was entering the cells and transcription of the GUS gene occurred but the frequency of integration into genomic DNA was very low. Narasimhulu *et al.*(1996) studied the early events involved with T-DNA transfer, transcription and integration into cells of tobacco and maize. They observed that the initial kinetics for the appearance of GUS transcripts was approximately equal for both species and that there was no major difference in T-DNA transfer, nuclear targeting or conversion to a double-stranded DNA form. However, while GUS transcripts were present in tobacco cells for at least 7 days, they dissappeared in maize

within 36 hr after inoculation. This result was an indication that the T-DNA was being lost or degraded in maize; whereas, in tobacco it was being integrated into the tobacco genome. Since transient expression in maize was equivalent to that of tobacco, they suggested the inability to efficiently transform maize with *Agrobacterium* does not involve the infection process but rather there is a block at the level of T-DNA integration into the genome. This block may be the same reason for the low frequency of stable transformation observed in asparagus using *Agrobacterium.*

Another potential reason for the low frequency of stable transformation could be genotype. *A. officinalis* cv. Rutgers 22 was used in this study because it is amenable to forming embryogenic cultures as determined in previous work in our laboratory and for its potential breeding value. Studies have shown that genotypes of the same species can vary widely in their response to transient and stable *Agrobacterium* transformation (Van Wordragen *et al.* 1991; 1992). Conner *et al.* (1988) investigated the *Agrobacterium* x genotype interaction in asparagus using four wild-type strains of *A. tumefaciens* and 17 asparagus genotypes. Only *A. tumefaciens* wild-type strain C58 produced tumors on five of the 17 genotypes. Moreover, *A. officinalis* cv. CRD 157 produced the majority of tumors. There is the possibility that Rutgers 22 is recalcitrant to T-DNA integration and that other genotypes may have yielded a higher frequency of transgenic tissues.

The parameters evaluated in this study comprised those known to be most influencial for successful *Agrobacterium* transformation. Among those, the most important being *Agrobacteruim* strain. The *Agrobacterium* strains that were used corresponded either to those that have successfully produced transgenic asparagus tissues or have potential merit in asparagus transformation. In previous studies in which transgenic plants were produced, strains corresponding to GV3101(pMP90) (C58C1:pGV3850::1103neo) (Bytebier *et al.* 1987; Conner *et al.* 1988) and GV3101(pGV2260) (Delbreil *et al.* 1993) were used. Strain EHA105, derived from the wild-type A281, carries virulence genes from pTiBo542 and is considered to be hypervirulent (Hood *et al.* 1986). A281 was reported to be ineffective in forming tumors on asparagus spears(Conner *et al.* 1988) but has been effective in transforming other monocots (Raineri *et al.* 1990; Dong *et al.* 1996). Finally, strain Ach5 is the wild-type progenitor to LBA4404. Ach5 has the ability to aggregate asparagus cells which was used as an indication of plant cell attachment (Draper *et al.* 1983). Cell attachment is one of the first steps in the *Agrobacterium* transformation process. Aggregation by Ach5 actually exceeded that caused by C58 strains in that study.

The strain of *Agrobacterium* used did prove to be an important factor in this study. The strains that produced transgenic plants in studies by Bytebier *et al.* (1987) and Conner *et al.*(1988) were equivalent to GV3101(pMP90).

GV3101(pMP90) produced the greatest amount of transient GUS expression along with EHA105. Delbreil *et al.* (1993) successfully produced transformed embryogenic cell cultures of asparagus with GV3101(pGV2260). However, this C58-derived strain was not found to be effective in this study. Since transient GUS expression was not significantly different between EHA105 and GV3101(pMP90) and there was no significant amount of transgenic tissues produced, it is difficult to assertain which strain is best.

At the time of this study, kanamycin was the only selective agent that had been used to select for transgenic asparagus tissues(Bytebier *et al.* 1987; Conner *et al.* 1988; Delbreil *et al.* 1993; Mukhopadhyay and Desjardins 1994). Few transgenic plants were produced in those studies. Since the efficiency of selection is an important factor in the sucessful recovery of transgenic plants (Lindsey and Jones 1990), evaluating selective agents other than kanamycin was warranted. As a potential alternative to kanamycin, G418 (geneticin) was tested because it is also an aminoglycoside antibiotic that is detoxified by the NPTII gene and has been shown to be more effective than kanamycin in some dicots and most monocots(Norelli and Aldwinckle 1993; Wilmink and Dons 1993; Laparra *et al.* 1995). Previous asparagus transformation studies selected tissues using up to 100 mg/l kanamycin. This level was found to be insufficient for suitably inhibiting growth of embryogenic cells and escapes could be anticipated.

The asparagus cells were approximately twice as sensitive to G418 as they were to kanamycin. It is believed that the increased sensitivity to G418 is caused by more effective binding to the ribosomes compared to kanamycin (Wilmink and Dons 1993). The asparagus cells exhibited the greatest amount of sensitivity to glufosinate. Unlike the aminoglycosides that suppress protein synthesis, glufosinate is an herbicide that inhibits glutamine synthase and causes rapid cell death from the accumulation of ammonia in the cells (Tachibana *et al.* 1986). Although G418 and glufosinate were determined to be more effective than kanamycin in inhibiting growth, the actual effect of the selective agents on transformation and regeneration of transgenic plants could not be determined since a population of transgenic plants was not produced.

The other parameters that were tested in this study aided in T-DNA transfer and transient GUS expression. Determining the length of cocultivation provided information as to when the greatest amount of GUS expression could be detected to aid in scoring the experiments. This information was applied directly to timing of selection of transformed cells. The proper inoculum density is a balance between the size of the inoculum required for high levels of DNA transfer weighed against deleterious effects fo bacterial growth on the inoculated tissues (Lindsey and Jones 1990). Transient GUS expression was the greatest at the lowest densities tested which may be an indication that inoculum densities above 10^8

might be deleterious. At densities of 5 x 10^9 cfu/ml and above, the cells were observed to be brownish in appearance indicating that the lower expression may have been due to deleterious conditions. Part of the transformation process involves the attraction of *Agrobacterium* to the cell surface or wound site and the activation of the *vir*-genes by phenolic signal molecules. The most commonly used compound for inducing the *vir*-genes prior to transformation to aid T-DNA transfer is acetosyringone (Stachel *et al.* 1985). Asparagus does produce a moderate amount of phenolic signal molecules compared to other monocots which may be an explanation why it can be naturally infected (Messens *et al.* 1990). However, while acetosyringone did significantly enhance T-DNA transfer and transient GUS expression, it did not have an effect on T-DNA integration.

In this study, *Agrobacterium tumefaciens* had the ability to transform asparagus as exhibited by transient GUS expression assays. However, an efficient transformation system that routinely yields a high frequency of transgenic plants will require a greater understanding of the requirements for T-DNA integration into the asparagus genome.

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SUMMARY AND RECOMMENDATIONS

Protoplast Culture

I found that genotype and auxin source influences asparagus protoplast culture. In addition, protoplasts derived from a highly embryogenic cell source can undergo direct somatic embryogenesis under the proper conditions. Upon initiating protoplast culture with an untested genotype, embryogenesis should be attempted with both 2,4-D and NAA. If both auxins produce embryogenic cultures then preference should be given to the NAA cultures due to the lower frequency of somaclonal variation. It is very important to select embryogenic material, such as clusters of somatic embryos, for initiating suspension cultures. This will ensure that the population of donor cells are as embryogenic as possible prior to protoplast isolation. If non-embryogenic callus is used for this purpose, the suspension cell population will be more heterogenous and contain fewer embryogenic cells. For protoplast culture, the X-plate system described in chapter two is very effect. For NAA derived protoplasts, it would be useful to examine using levels of NAA far below 50 µM for protoplast culture. The lack of NAA in the protoplast culture medium allowed for the development of direct somatic embryos from NAA-derived protoplasts, however, a low level of NAA may be beneficial for protoplast culture with other genotypes. Using 50 µM was apparently toxic in our study but may expedite cell divisions at lower levels.

Transformation

The production of transgenic asparagus tissues and plants via *Agrobacterium*-mediated transformation has occurred at a very low frequency in previous reports. This study attempted to develop an efficient *Agrobacterium*-mediated transformation protocol that was based on using embryogenic cells to expedite regeneration of transgenic plants via somatic embryogenesis. Unfortunately, the frequency of transformation was also very low in our study. Transformation of asparagus should be pursued until an efficient and genotype independent system is developed. The following are recommendations for further studies. The embryogenic colonies were placed on EM medium for embryo development and maturation directly following inoculation. An alternative to this is to place the inoculated tissues onto a medium containing a selective agent (glufosinate) that promotes the development of embryogenic callus. This would allow the transformed regions of the cell masses to proliferate instead of depending on single transformed cells to give rise immediately to transformed somatic embryos. A second recommendation is to inoculated smaller cell colonies. Smaller or less developed colonies are rapidly growing, potentially more transformation competent and transgenic regions on such colonies are easily selected for. A final recommendation is to use direct-gene-mediated transformation in the form of the gun. T-DNA is associated with proteins from the *Agrobacterium* virulence genes that may

be recognized by the cell causing degradation or blocking integration into the asparagus genome. Direct-gene-mediated transformation is not associated with any proteins that could be recognized by the cell. Although transformation via biolistics is less efficient than with *Agrobacterium*, use of the gene gun is warranted for asparagus and may not be as genotype dependent.