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DEVELOPMENT OF *FUSARIUM* RESISTANCE OF ASPARAGUS VIA TISSUE CULTURE AND GENE TRANSFORMATION

By

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ABSTRACT

DEVELOPMENT OF *FUSARIUM* RESISTANCE OF ASPARAGUS VIA TISSUE CULTURE AND GENE TRANSFORMATION

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Asparagus officinalis L. is an economically important vegetable crop that is produced worldwide. However, a disease syndrome known as asparagus decline, primarily caused by *Fusarium oxysporum* and *F. proliferatum*, decreases the annual yields of asparagus over time, and is found throughout the asparagus growing regions of the world. The use of conventional methods including chemical and cultural methods, and traditional breeding of resistance in the host for controlling *Fusarium* spp. has been limited. The objective of this study was to explore the development of *Fusarium* resistance of asparagus via tissue culture and gene transformation, and determine the genetic base of the resistance. A procedure of isolation, culture and plant regeneration of callus-derived protoplasts of *Asparagus officinalis* cv. Lucullus 234 was developed. Protocols were also developed for vegetative micropropagation and somaclone production of 'Lucullus 234'. One hundred and twenty somaclones screened for resistance to *F. oxysporum* and *F. proliferatum* in the greenhouse had significantly increased levels of resistance over the vegetatively micropropagated 'Lucullus 234' parental plants. Two somaclonal lines demonstrating increased resistance from the first cycle of screening, R7 and R4, were

rated as highly resistant compared with the parental plants when rescreened with *F. oxysporum* in the greenhouse after they were vegetatively micropropagated. Cytogenetic studies showed that R7 and R4 were diploid with normal chromosomal numbers (2n=20) as 'lucullus 234' parental cultivar (2n=20). The genomic fingerprints obtained by randomly amplified polymorphic DNAs (RAPDs) technology indicated that resistant somaclonal lines, R7 and R4, may have carried DNA sequence mutations, as their DNA sequences varied from the 'Lucullus 234' parental plants. Spear segments of 'Lucullus 234' were transformed using the binary vector *Agrobacterium tumefaciens* LBA4404/pBI121 containing an anti-bacterial and -fungal gene (Shiva-1) encoding a lytic peptide. A single transgenic plant (T11) was obtained. This plant displayed GUS activity, and Southern blot suggested the presence of two copies of the Shiva-1 gene integrated into the genome of T11. An in-vitro antibacterial assay showed that T11 significantly inhibited the growth of *E. coli*, indicating possible production of Shiva-1 peptide in T11. The vegetatively micropropagated plants of T11 showed a significant increase in resistance to *F. oxysporum* in the greenhouse in comparison with vegetatively micropropagated nontransgenic plants.

To

My parents, whose unselfish love supported me through my education.

My husband, for his endless love, understanding and support.

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NAA	alpha-naphthaleneacetic acid
2,4-D	2,4-dichlorophenoxyacetic acid
6-BAP	6-benzylaminopurine
IBA	indolebutyric acid
IAA	indoleacetic acid
kb	kilobases

INTRODUCTION

Botanical aspect

The genus *Asparagus*, a member of the family Liliaceae, has about 150-300 herbaceous and woody perennial species widely grown in the temperate and tropical regions of the world (Lawrence, 1982). *Asparagus officinalis* L. is native to the Orient and to the east of the Mediterranean genecenter, and has been the only species cultivated as an edible vegetable plant for over 2,000 years. *A. officinalis* is dioecious with 2n = 20 (Jessop, 1966) and a sex ratio of 1:1; however, male plants have both staminate and hermaphroditic flowers (Reuther, 1984). Female plants are homogametic XX, male plants are heterogametic XY, and hermaphroditic flowers arise on male (XY) plants due to a rare mutation. The male with hermaphroditic flowers are andromonoecious plants which can be cross- or self-fertilized (Wircke, 1979; Reuther, 1984). Andromonoecious plants produce two genotypes: XY male and YY super-male after self-fertilizing. Crossing supermales with females produces all-male plants which are most desirable because of their higher yield, vigor, and longevity (Ellison *et al.*, 1960). In addition, increase of disease resistance and drought tolerance are associated with male plants (Ellison, 1986). Andromonoecious plants can make up 10 to 20% of some asparagus populations (Wircke, 1979).

Importance and problems of asparagus production

A. officinalis is an economically important vegetable crop in over 17 countries (Reuther, 1984). The United States produces 45,870 ha with California (17,180 ha), Washington (13,640 ha), Michigan (9,320 ha), and New Jersey (870 ha) as the primary producers, and is the most important country in the world for asparagus production (Desjardins, 1992). Asparagus is one of the most important vegetable crops in Michigan (Kindinger, 1987) with an average annual value of over 15 million dollars (Fedewa and Pscodna, 1993). It is a perennial vegetable crop, and properly maintained asparagus fields should remain productive for 20 or more years with a yield of 2-3,000 lb per acre or more. However, today's asparagus fields are being removed from production after only 8 to 15 years due to sparse stands and small spear size, resulting in low yields (Takatori and Souther, 1978). This problem is due to a disease syndrome known as "asparagus decline" which causes loss in longevity and productivity of established fields, difficulty in replanting asparagus where asparagus was previously grown, and thereby decreases in annual yields of asparagus over time. The disease is found throughout the world (Cohen and Heald, 1941; Graham, 1955; Grogan and Kimble, 1959; Van Bakel and Kerstens, 1970; Endo and Burkholder, 1971). Most field are planted with about 10,000 crowns per acre, but often 50% are lost in the first five years after planting due to "asparagus decline". In Michigan asparagus fields, the average crown population was 3153 per acre from 1978 to 1979, and this represented a 70% reduction in crown survival (Hodupp, 1983). In 1978, the average yield for Michigan was 1,500 lb/A; but in 1981 the state average was only 900 lb/A (Bates *et al.*, 1987). Asparagus decline also reduced the acres cultivated. Asparagus production has decreased from 30,000 acres to

less than 1000 over the last 25 years in New Jersey (Herner and Vest, 1974), from 44,000 acres in 1974 to 28,000 in 1978 in California (Takatori and Souther, 1978) and from 500 ha in 1963 to 340 in 1970 in the Netherlands (Van bakel and Kerstens, 1970).

Factors contributed to the problems

Abiotic factors such as environmental stresses (nutrient imbalance, low soil pH and soil moisture), physical stresses (defoliation by asparagus beetle, culture practice and allelopathic substances produced by asparagus tissue), and biotic factors such as asparagus virus I and II, and *Stemphylium vesicarium* have been implicated in the decline (Kitahura *et al.*, 1972; Laufer and Garrison, 1977; Yang, 1982, 1985; Falloon *et al.*, 1984; Young, 1984; Evans and Stephens, 1985; Shafer and Garrison, 1986).

However, the most important factors associated with "asparagus decline" are *Fusarium oxysporum* f. sp. *asparagi* Cohen and Heald and *F. proliferatum* (T. Matsushima) Nirenberg (syn. *F. moniliforme* J. Sheld.)(Cohen and Heald, 1941; Graham, 1955; Grogan and Kimble, 1959; Van Bakel and Kerstens, 1970; Endo and Burkholder, 1971). *F. oxysporum* and *F. proliferatum* are important pathogens of major crops throughout the world. They are chitinous and facultative parasites which colonize living and non-living host tissue and may invade non-host tissue (Alexander, 1961; Hendrix *et al.*, 1958). They are able to form chlamydospores or other resting structures which can survive in soil for many years. These characteristics make them especially persistent once they are established (Nelson *et al.*, 1981). *F. oxysporum* causes a vascular wilt which inhibits water uptake and carbohydrate transport within the plant. *F. proliferatum* is primarily a root-rotting organism. Asparagus plants infected by either pathogen show

similar above-ground symptoms; the ferns are yellowed, stunted, wilted, and may die in different stages of development (Cohen, 1946; Endo and Burkholder, 1971). *F. oxysporum* causes root rotting and the symptoms of vascular discoloration within the stem, root and crown (Endo and Burkholder, 1971). *F. proliferatum* causes more extensive dry crown rot and brown stem pith discoloration but no vascular discoloration (Cohen, 1946).

Conventional controls of *Fusarium* and their limitations

Conventional methods of controlling *Fusarium* spp. are limited. Chemical treatments, including crown dips and foliar sprays, have not been successful (Stephens *et al.*, 1991). Fumigation does not offer long-term effectiveness because of the perennial nature of the crop (Lacy, 1979). Further, *Fusarium* spp. are ubiquitous and may reinfest the soil following fumigation and rapidly colonize young asparagus plants in the field (Damicone and Manning, 1985). Cultural control such as incorporation of cruciferous residues reduces *Fusarium* population, but does not eliminate the disease (Stephens and Sink, 1992). To date, no *Fusarium* resistant varieties have been developed (Takatori and Souther, 1978; Ellison, 1986).

Alternative strategies to control *Fusarium*

The most successful strategy for Fusarium wilt control in other vegetable crops has been the development of resistant varieties (Mace *et al.*, 1981). Possibilities exist for developing *Fusarium* resistant varieties in asparagus. A cultivar of *A. officinalis*, Lucullus 234, had the highest resistance to virulent Michigan isolates FOA10 of *F.*

oxysporum and FM12 of *F. proliferatum* among 90 cultivars and breeding lines of this species tested (Stephens *et al.*, 1989). Two ornamental cultivars of *Asparagus densiflorus* (Kunth) Jessop (cvs. Sprengeri and Myersii) were resistant to *F. oxysporum* and *F. proliferatum* in greenhouse studies (Stephens *et al.*, 1989). Further, it was suggested that *A. densiflorus* cv. Sprengeri was immune to *F. oxysporum* f. *asparagi* in a laboratory study (Lewis and Shoemaker, 1964). However, sexual crosses of *A. officinalis* with the resistant species, *A. densiflorus* 'Sprengeri' have been unsuccessful, probably due to incompatibility barriers (Elmer *et al.*, 1989). In addition, using conventional methods, *A. officinalis* has low regenerative potential (Reuther, 1984), and the development of new cultivars requires many years because of its perennial nature. It is likely that genetic diversity among the asparagus cultivars in North America is low (Gleason and Cronquist, 1963; Luzny, 1979; Ellison, 1986). In this context, tissue culture and micropropagation were investigated as a possible means of locating resistant lines.

Isolated protoplasts are a unique tool for genetic manipulation of plants such as regeneration of the entire plant from protoplasts, somatic fusion between sexually incompatible plant species and incorporation of interesting DNA into protoplasts. Plant regeneration from protoplasts has enabled investigators to create new protoplast-derived somaclones as novel genetic sources. Protoplast fusion has provided somatic hybrids/cybrids at interspecific and intergeneric levels for widening the pools of germplasm in crop improvement programs. In addition, the storage of protoplasts through immobilization and cryopreservation are of great importance, especially for the pharmaceutical industry (Bajaj, 1989). Since the first report on the regeneration of

complete plants from protoplasts (Takebe *et al.*, 1971), remarkable progress has been made and a number of commercially important crops such as potato, tobacco, tomato, rice, maize, cucumber and eggplant etc. are routinely regenerated (Bajaj, 1989).

Genetic variability was first reported in cultured plant cells in 1967 (Murashige and Nakano, 1967). Variation among plants regenerated from tissue culture has been termed "somaclonal variation" (Larkin and Scowcroft, 1981). Variation has been detected in cultured cells, especially on periodical subculturing for various morphological and genetic changes, such as polyploidy, aneuploidy, chromosome breakage, deletion, translocation, gene amplification, inversions, mutations, etc. (Nagl, 1972; Meins, 1983; D'Amato, 1985). Molecular and biochemical changes occurred in the DNA (Berlyn,1982; Cullis, 1983), enzymes (Brettell *et al.*, 1986), gliadin (Cooper *et al.*, 1986), etc.. Somaclonal variation was first proposed as a novel source of agriculturally useful variation for asexually propagated crops such as sugarcane (Heinz *et al.*, 1977) and potato (Shepard *et al.*, 1980). Somaclonal variation has been used to recover genetic variability at high frequency in a number of crop varieties, and offers an alternative to mutation breeding. Somaclones have shown resistance/tolerance to pathogenic fungi (Forougi-Wehr *et al.*, 1986; Rines, 1986; Meulemans *et al.*, 1987), bacteria (Thanutong *et al.*, 1983; Hammerschalg, 1986; Sun *et al.*, 1986), viruses and nematodes (Wenzel and Uhrig, 1981), phytotoxins (Gengenbach *et al.*, 1977), herbicides (Chaleff and Ray, 1984), and salt (Nabors *et al.*, 1980; Bajaj and Gupta, 1987). In addition, high-yielding (Ogura *et al.*, 1988), rich in protein (Schaeffer and Sharpe, 1987) and sugar contents (Liu and Chen, 1976), and male sterility (Ling *et al.*, 1987) have been obtained in somaclones of different crops. Important agricultural crops such as wheat, rice, maize, potato,

sugarcane, brassica. etc. have already yielded positive results to the extent of new cultivars being released (Bajaj, 1990).

The soil bacterium, *Agrobacterium tumefaciens*, can to infect most dicotyledonous plants at wounding sites (Braun, 1978, 1982) and induce formation of tumors, called crown gall, via the transfer of a plasmid it contains, called the Ti-plasmid (Zaenen *et al.*, 1974; Van Larebeke *et al.*, 1974; Watson *et al.*, 1975). When the bacterium comes in contact with a wounded plant cell, the Ti-plasmid is transferred from the bacterium into the cell. A small segment of the plasmid, T-DNA, is transferred from the plasmid to the nucleus of the plant cell, and becomes integrated into the plant nuclear genome (Chilton *et al.*, 1977, 1978; Willmitzer *et al.*, 1980). This organism became the main target for development of a plant transformation technique. *Agrobacterium*-mediated gene transfer is now a powerful tool for introducing foreign genes into many plant species to improve agricultural crops by increasing of resistance to pathogenic viruses (Nelson *et al.*, 1988; Hoekema *et al.*, 1989), fungi (Broglie *et al.*, 1991) and bacteria (Destefano-Beltran *et al.*,1990). Traditionally it has been considered that *Agrobacterium*-mediated transformation was limited to dicotyledonous plant species; however it is now becoming increasingly clear that *Agrobacterium* can transfer DNA to cells of monocotyledonous plants such as asparagus (Hernalsteens *et al.*, 1984; Bytebier *et al.*, 1987; Delbreil *et al.*, 1993), rice (Raineri *et al.*, 1990), wheat (Mooney *et al.*, 1991) and corn (Graves and Goldman, 1986).

It was possible that highly disease resistant asparagus cultivars could be derived from selection of somaclonal variation among plants regenerated from protoplasts of 'Lucullus 234' in response to *F. oxysporum* and *F. proliferatum*, *Agrobacterium*-mediated or direct

gene transfer of asparagus with foreign fungal resistant genes such as Shiva-1 gene, and protoplast fusion of 'Lucullus 234' with the resistant *A. densiflorus* 'Sprengeri' or 'Myersii'.

Objectives of this study

The overall objective of these studies was to develop *Fusarium* resistance of asparagus via tissue cultures and gene transformation, and determine the genetic base of the resistance. To approach this goal, the following studies were conducted 1) to develop a procedure to isolate, culture and regenerate plants from callus-derived protoplasts of 'Lucullus 234', 2) to develop protocols for vegetative micropropagation and somaclone production of 'Lucullus 234', 3) to screen protoplast-derived somaclones of 'Lucullus 234' for resistance to *F. oxysporum* and *F. proliferatum*, and characterize the genetic base of the *Fusarium* resistant somaclones by randomly amplified polymorphic DNAs (RAPDs) technology and cytogenetic study, and 4) to develop *Agrobacterium*-mediated gene transformation of asparagus ('Lucullus 234') with antifungal gene 'Shiva-1', verify the transformed plants and evaluate the transformed plants in response to the most virulent Michigan isolate of *F. oxysporum*, FOA50.

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CHAPTER I

Optimal Conditions for Growth and Plant Regeneration of Asparagus (*Asparagus Officinalis* L. cv. Lucullus 234) from Callus-Derived Protoplasts

ABSTRACT

Efficient protocol was developed for plant regeneration from callus-derived protoplasts of *Asparagus officinalis* L. cv. Lucullus 234. In addition, the effects of protoplast culture media on protoplast growth were investigated as well as types of culture, and osmotic conditions or hormone concentrations in liquid media of bead culture. Plating efficiency and colony formation differed significantly among different protoplast culture media and types of culture. Osmotic conditions and hormone concentrations of liquid media had the greatest influence on plating efficiency and colony formation in bead culture. Protoplasts grew best in bead culture with a solid modified Kao & Michayluk protoplast culture medium (KM) supplemented with 0.5 mg l^{-1} NAA, 0.5 mg l^{-1} 2,4-D, 0.5 mg l^{-1} kinetin, and 0.6% agarose (KM6), and in a similar liquid medium differing in sugar content, having 0.18 M sucrose and 0.18 M mannitol (A8). An average plating efficiency and colony formation (19.1% and 15.5% respectively) was obtained one week after isolation in bead culture with the KM6 and A8 media. The highest average shoot regeneration of 92.3% was obtained with a Murashige & Skoog

medium (MS) containing 0.125 mg l^{-1} NAA, 0.125 mg l^{-1} 2,4-D, 0.25 mg l^{-1} 6-BAP and 3% sucrose. Plants have been regenerated after shoots were transferred into hormone-free MS medium. Plants were transplanted in the greenhouse.

INTRODUCTION

Asparagus (*Asparagus officinalis* L.) is an important vegetable crop and produced worldwide (Desjardins, 1992). However, a disease syndrome known as asparagus decline, primarily caused by *Fusarium oxysporum* f. sp. *asparagi* Cohen and Heald and *F. proliferatum* (T. Matsushima) Nirenberg, decreases annual yields of asparagus over time, and is found throughout the world (Cohen and Heald, 1941; Graham, 1955; Grogan and Kimble, 1959; Van Bakel and Kerstens, 1970; Endo and Burkholder, 1971). Attempts to sexually cross *A. officinalis* with the resistant ornamental species, *A. densiflorus* 'Sprengeri' or 'Myersii' have been unsuccessful, probably due to incompatibility barriers (Elmer et al., 1989). Also, chemical and cultural controls of *Fusarium* spp. in asparagus fields have not been effective (Lacy, 1979; Stephens et al., 1991; Stephens and Sink, 1992). Tissue culture techniques offer new approaches to develop *Fusarium* resistant asparagus from a cultivar of *A. officinalis*, Lucullus 234, which had the highest tolerance to *F. oxysporum* and *F. proliferatum* compared to 90 cultivars and breeding lines of this species (Stephens et al., 1989). Protoplast fusion provide the only current means of obtaining somatic hybrid plants to overcome sexual incompatibility barriers . Protoplasts have been used for direct uptake of desired DNA or chromosomes by chemically mediated, electroporation, and microinjection methods into target plants (Lal, 1990). In addition, plant regeneration from protoplasts offers

novel somaclonal variation for crop improvement such as increase of disease resistance (Bajaj, 1990)). However, to pursue these approachs, an efficient protoplast regeneration protocol is necessary.

The division of protoplasts from *Asparagus officinalis* and their growth and differentiation were reported by Bui Dang Ha and Mackenzie (1973). The same group regenerated plants of *A. officinalis* through callus cultures derived from protoplasts (Bui Dang Ha and al., 1975). Chin et al. (1988) reported culture of droplets containing asparagus cells and protoplasts on polypropylene. Kong and Chin (1988) regenerated plants of *A. officinalis* using agarose on polypropylene membrane. Elmer et al. (1989) reported plant regeneration from callus-derived protoplasts of *A. officinalis*. Plating efficiency (the numbers of cells divided / total protoplasts plated) was low, and cell division, colony formation and callus formation were slow in the above studies of protoplast isolation, culture and plant regeneration of different cultivars of *A. officinalis*. In addition, only three of these investigations reported plant regeneration (Bui Dang Ha et al., 1975; Kong and Chin, 1988; Elmer et al., 1989). Bead culture is used successfully in protoplast culture of other plant species to increase plating efficiency (Shillito et al., 1983; Furze et al., 1987), but has not been used in protoplast culture for asparagus. Further, the effects of osmotic conditions and hormone concentration in liquid media in bead culture on protoplast growth have rarely been reported.

This chapter reports the development of a new protocol for culture, isolation and plant regeneration of callus-derived protoplasts of asparagus cultivar Lucullus 234. In addition, the objectives were to study the effects of: 1) liquid, argrose-layer and bead culture on plating efficiency and colony formation of protoplasts; 2) different types (2,4-

D, NAA, kinetin, zeatin and 6-BPA) and concentrations of plant growth regulators on plating efficiency and colony formation; 3) osmotic conditions and hormone concentrations in liquid medium of bead culture on plating efficiency and colony formation; 4) different cytokinins (6-BAP, zeatin and kinetin) on shoot differentiation.

MATERIALS AND METHODS

Plant material Shoots from 2- to 3-year old greenhouse-grown plants of *A. officinalis* cv. Lucullus 234 were surface-sterilized for 30 m in a 20% (v/v) aqueous bleach solution (5.25% sodium hypochlorite, Big Chief Bleach, Patterson Laboratories, Detroit MI) plus 3 ml per liter of Tween-20 (Sigma Chemical Co., St. Louis MO). Shoots were rinsed three times with sterile distilled water and placed in 100 x 15 mm plastic Petri dishes containing MSF medium (Elmer et al., 1989). After incubation in the dark at 27°C for one month, calli were excised from the shoots and subcultured every four weeks.

Isolation of protoplasts Callus (2.5 g) was removed from subculture, sliced thinly with a new scalpel blade, and placed in 30 ml of enzyme solution (Elmer et al., 1989) of 1% Cellulysin, 0.2% Macerase and 1% Rhozyme dissolved in CPW solution (Cocking and Peberdy, 1974) at pH 5.6 sterilized by passing through a 0.22μm filter. Protoplasts were released during a 16 to 17 h digestion period in the dark at 27 to 28°C on a gyratory shaker at 30 rpm. The protoplasts were purified using the modified protocol of Elmer et al. (1989). Protoplasts were collected through a 61 um nylon sieve separating undigested clumps of callus. Protoplasts were washed three times with CPW solution

plus 0.7 M mannitol at pH 5.85 (CPW wash solution) and centrifuged at 70 g for 5 m. Protoplasts were floated in CPW solution with 21% sucrose (pH 5.85) on top of which 3 ml of CPW wash solution was layered and centrifuged at 150 g for 5 m. After centrifugation, protoplasts were separated from undigested cells at the interface between the solutions. Protoplasts were collected, suspended in CPW wash solution, counted with a hemacytometer, centrifuged at 70 g for 5 m and diluted to 5×10^4 ml^{-1} for culturing in different protoplast culture media.

Culture of protoplasts The isolated protoplasts were cultured at a density of 5×10^4 ml^{-1}. Several factors were tested for their effects on plating efficiency and colony formation: types of culture (liquid, agarose layer, and bead cultures); growth regulators for liquid and agarose layer cultures; and osmotic condition and hormone concentrations of liquid media of bead culture.

Six different liquid protoplast media were tested for culturing in liquid or on agarose layer cultures. These media were based on a modified Kao & Michayluk protoplast culture medium (KM) containing 0.05 M sucrose, 0.05 M glucose and 0.7 M mannitol (Elmer et al., 1989). Hormone content of the six media were (mg l^{-1}): KM1= 2,4-D, 1 + 6-BAP, 0.5; KM2= 2,4-D, 1 + kinetin, 0.5; KM3= NAA, 1 + 6-BAP, 0.5; KM4= NAA, 1 + kinetin, 0.5; KM5= NAA, 0.5 + 2,4-D, 0.5 + 6-BAP, 0.5, and KM6= NAA, 0.5 + 2,4-D, 0.5 + kinetin, 0.5. Liquid cultures had 3 ml of protoplast suspension in liquid media plated onto 60 x 15 mm plastic Petri dishes. Agarose layer cultures had 3 ml of protoplast suspension in liquid media over 3 ml of the same media solidified with 0.4% agarose (Sea Plaque LMT, FMC Corp., Rockland ME). At weekly

intervals, 0.5 ml of the initial liquid protoplast culture media was removed and substituted with 0.5 ml of replacement media. Replacement media differed from the original media in sugar content (0.18 M sucrose and 0.18 M mannitol) and did not contain auxins. The plates were incubated at 27°C in the dark for 1 week followed by 1 week under 2 to 3 $\mu Em^{-2}s^{-1}$ light with 16 h photoperiod. The plates were maintained for an additional 3 weeks at 27°C with 40 $\mu Em^{-2}s^{-1}$ light and a 16 h photoperiod. All light was provided by cool-white fluorescent lamps.

For studies on effects of osmotic conditions of liquid medium in bead culture, KM6 was chosen as the solid medium in which 0.6% agarose was added for bead culture. Osmotic conditions of liquid medium in bead culture affected protoplast growth. This was investigated by comparing A8 liquid medium to KM6 liquid medium (control). A8 liquid medium differed from liquid medium KM6 (800 mOsmol/kg H_2O) in sugar content, having 0.18 M sucrose + 0.18 M mannitol (360 mOsmol/kg H_2O). The bead cultures were prepared according to the method of Shillito et al. (1983). Protoplasts were suspended in molten KM6 solid medium and 4 ml was plated onto 60 x 15 mm plastic Petri dishes. After solidification of the agarose (25-30 min), the agarose was then cut into 6 pieces and transferred to 30 ml of A8 and KM6 liquid medium in 100 x 20 mm plastic Petri dishes. Ten ml of initial liquid protoplast culture medium were removed and replaced with 10 ml of original fresh liquid medium every week for all bead cultures. The plates were placed on a gyratory shaker (55 rpm) at 27°C in the dark.

To study effects of hormone concentration of liquid medium of the bead culture on protoplast growth, A8 liquid medium was chosen because it was found have better plating efficiency than KM6 liquid medium for bead culture. KM6 medium was used as the

solid medium. Then, three liquid media similar to A8 medium but differing in growth regulator concentration were tested in bead culture. The three media were: A7, containing NAA, 2,4-D and kinetin at concentrations of 0.25 mg l^{-1}; A8, containing NAA, 2,4-D and kinetin at concentration of 0.5 mg l^{-1}, and A9, containing NAA, 2,4-D and kinetin at concentration of 1.0 mg l^{-1}. The bead cultures were prepared and maintained using the methods described previously.

For comparison of liquid, agarose layer, and bead culture, KM6 medium was selected as the protoplast culture medium for all cultures, and A8 liquid medium was used as both liquid medium for bead culture and replacement medium for all cultures. These three cultures were prepared using the methods described previously. At weekly intervals, 1 ml (for the liquid and agarose layer) and 10 ml (for bead culture) of the initial liquid protoplast culture medium were removed and replaced respectively with 1 ml and 10 ml of the fresh A8 liquid medium. The plates of bead culture were placed on a gyratory shaker (55 rpm) at 27°C in dark. The plates of liquid and agarose layer cultures were incubated at 27°C in dark.

Plating efficiency, is defined as the numbers of cells divided / total protoplasts plated. Colony formation, is defined as the numbers of cell colonies of more than three cells / total protoplasts plated. Plating efficiency and colony formation were recorded one week after isolation for bead culture. Plating efficiency was evaluated three weeks after isolation and colony formation recorded five weeks after isolation for liquid and agarose layer cultures. At least 500 cells of more than five random microscopic fields were examined. Protoplast yield is defined as the numbers of protoplasts per gram of callus. Protoplast yield was counted after flotation and the final wash (before plating in

protoplast culture media). All experiments were conducted as a completely randomized design with six replicate plates per treatment and were conducted at least three times. Values represent the mean of six replicate plates from at least three isolations. All the data in the tables were analyzed by ANOVA and Duncan's Multiple Range Test was used to separate means.

Plant regeneration of calli from single protoplasts Two to four weeks after initiation of the bead culture, calli formed from protoplasts were transferred to nine different shoot-inducing media and a hormone-free medium (control). The nine shoot-inducing media were made from three combinations of cytokinins and auxins at three concentrations. All media were based on Murashige & Skoog (MS) medium (1962) + 30 gl^{-1} sucrose at pH 5.85. The three hormone combinations were NAA:2,4-D:Kinetin, NAA:2,4-D:Zeatin, and NAA:2,4-D:6-BAP. Each hormone combination was at three concentrations of (mg l^{-1}) 0.125:0.125:0.25 (level 1), 0.25:0.25:1 (level 2), and 0:0:1 (level 3). MS medium without hormones was prepared as control. The plates were incubated at 27°C in the dark for three weeks, under 2 to 3 $\mu Em^{-2}s^{-1}$ for one week and 40 $\mu Em^{-2}s^{-1}$ with 16 h photoperiod for 1 week. Four weeks later, calli were transferred from shoot-inducing media to hormone-free media for shoot and plant regeneration at 27°C with 40 $\mu Em^{-2}s^{-1}$ light and a 16 h photoperiod. Plants were transferred to the greenhouse after they regenerated directly in hormone-free medium. All experiments for shoot induction contained at least six replicates (6 to 12 calli per Petri dish) per treatment and were conducted three times.

RESULTS AND DISCUSSION

Effects of different media on plating efficiency and colony formation in liquid and agarose layer cultures An average of protoplast yields from all the isolations was 1.23 x 10^6 protoplasts / gram of callus. Isolated protoplasts (Figure 1.1a) became nonspherical within 24 h after plating as their cell walls reformed. Two to nine percent of the surviving cells became oval and some budding occurred within 1 week. Three to seven days after plating, some cells entered into the first cell division, but did not continue to grow in liquid or agarose layer cultures. The first colony of four to six cells appeared 4 weeks after isolation. Both media and type of culture, and the interaction had a significant effect on plating efficiency (ANOVA, $P<0.05$). KM6 or KM5 media combined with agarose layer culture significantly increased plating efficiency over other media and culture types (Table 1.1). Only media significantly influenced colony formation. The type of culture and the interaction between media and type of culture did not significantly affect colony formation. The colony formation was significantly higher with KM6 medium combined with agarose layer culture in comparison to other media (Table 1.1). Hormone content and agarose in the protoplast media played an essential role in protoplast culture. By the combination with agarose layer culture, only the media in the presence of NAA (KM6 = KM basal + 0.5 mg NAA + 0.5 mg 2,4-D + 0.5 mg kinetin/l and KM5 = KM basal + 0.5 mg NAA + 0.5 mg 2,4-D + 0.5 mg 6-BAP/l) significantly increased cell division. KM6 stimulated sustained division. This observation is in contrast to Elmer at al. (1989), who obtained sustained division of asparagus protoplasts only in a similar medium without NAA (the same KM basal +

Table 1.1. Effects of different media and types of culture on plating efficiency and colony formation of asparagus protoplasts.

Protoplast medium	Type of culture	Plating efficiency (%)[1] (cells divided / total protoplasts plated)	Colony formation (%)[1] (colonies > 3 cells / total protoplasts plated)
KM 6	Agarose layer	1.38 A	1.27 A
	Liquid	0.29 C	0.43 B
KM 5	Agarose layer	0.91 AB	0.41 B
	Liquid	0.00 C	0.00 B
KM 4	Agarose layer	0.49 BC	0.00 B
	Liquid	0.18 C	0.00 B
KM 3	Agarose layer	0.24 C	0.00 B
	Liquid	0.14 C	0.00 B
KM 2	Agarose layer	0.14 C	0.07 B
	Liquid	0.14 C	0.00 B
KM 1	Agarose layer	0.06 C	0.00 B
	Liquid	0.03 C	0.00 B

[1]Means of 6 replicate plates from all four isolations. Plating efficiency was evaluated 3 weeks after isolation and colony formation was recorded 5 weeks after isolation. Means within a column with different letters are significantly different (Duncan's multiple range test, $P < 0.05$).

1mg 2,4-D + 0.5 mg 6-BAP/l). Elmer et al. concluded that NAA was not required for sustained division of asparagus protoplast, but they used different cultivars. However, the plating efficiencies (0.0% to 1.4%) and colony formation (0.0% to 1.3%) were very low, and few calli were obtained after 8 weeks of culture for all treatments investigated.

Effects of osmotic condition of liquid media on plating efficiency and colony formation in bead culture The first cell division occurred the second day after isolation (Figure 1.1b), and colony formation with four to twelve cells appeared after 3 days in the liquid medium A8 in bead culture (Figure 1.1c). Cell colonies grew up to 0.48 mm diam. (Figure 1.1d) within 1 week and developed into microcalli (1 mm diam.) within 2 weeks after isolation (Figure 1.2a). Average plating efficiency and colony formation of A8 liquid medium (19.1% and 15.5% respectively) was significantly greater than those of KM6 liquid medium (control) (1.1% and 0.2% respectively)(Table 1.2). With KM6 medium as the liquid medium (control) in the bead culture, cell division began 2 to 4 days after isolation and colony formation occurred 1 week after isolation. Although a few divided cells and colonies were present 1 week after isolation, they did not continue to grow and did not form calli (Table 1.2). In addition, after bead cultures were maintaining in KM6 liquid medium for 3, 5 and 7 days, the KM6 liquid medium were totally replaced by 30 ml of A8 medium. The protoplasts did not continue to grow and died although a few cell divided. The high osmotic condition of KM6, when used as the liquid medium of bead culture, significantly decreased the plating efficiency and colony formation, and completely inhibited any subsequent callus formation (Table 1.2). Although the liquid A8 medium had 0.18 M mannitol and 0.18 M sucrose without

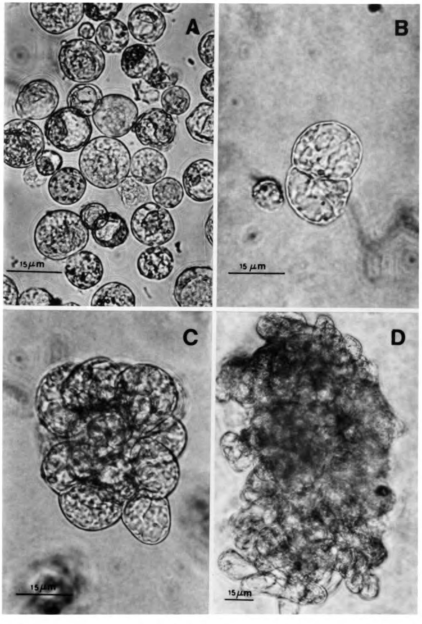

Figure 1.1. Development of *A. officinalis* cv. Lucullus 234 from callus-derived protoplasts to colony in bead culture (a-d). (a) Asparagus protoplasts immediately after isolation from calli cultured in medium MSF; (b) first division of protoplasts embedded in agarose of bead culture 1 day after isolation; (c) a colony of about 10 cells derived from protoplasts embedded in agarose of bead culture 5 days after isolation; (d) a cell colony derived from protoplasts embedded in agarose of bead culture 1 week after isolation.

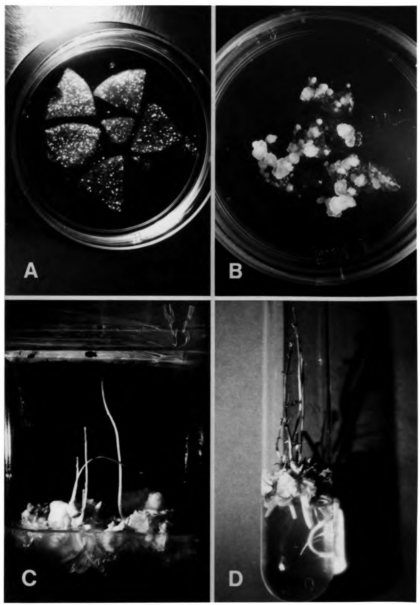

Figure 1.2. Development of *A. officinalis* cv. Lucullus 234 from colony to plants (a-d). (a) microcalli derived from protoplasts embedded in agarose of bead culture 2 weeks after isolation; (b) calli derived from protoplasts on shoot-inducing medium with 6-BAP at level 1 one week after initiation of bead culture; (c) callus regenerated from protoplasts with shoots and plantlets in hormone-free medium; (d) plant regeneration from protoplast-derived calli in hormone-free medium.

Table 1.2. Effects of sugar content of liquid media on the plating efficiency and colony formation in bead culture.

Liquid media	Osmolarity (mOsmol/kg H^2O)	Plating efficiency (%)[1] (cell divided / total protoplast plated)	Colony formation (%)[1] (colonies > 3 cells / total protoplasts plated)	1^{th}cell division (days)	Colony formation (days)	Microcalli formation (days)
A8	360	19.12 A	15.52 A	2	3	10
KM6	800	1.08 B	0.19 B	2-4	7	no microcalli

[1]Means of 6 replicate plates from all three isolations. Means within a column with different letters are significantly different (ANOVA, $P < 0.05$). Plating efficiency and colony formation was evaluated 1 week after isolation.

glucose, the presence of glucose (0.05 M) in liquid KM6 medium was not considered to have inhibitory effect on protoplast growth because amount of glucose was only 6% of total sugar in the medium. Also glucose is widely used in protoplast culture including asparagus protoplast culture and may be the preferred carbon source for most protoplasts (Evans and Bravo, 1983; Elmer et al., 1989). These results indicate that the high osmotic conditions, which is considered to be necessary to prevent bursting of protoplasts during early culture (Evans and Bravo, 1983), inhibited cell division later and eventually result in cell death. Bead culture permits use of lower concentrations of sugar in the liquid medium, thereby reducing the osmotic conditions immediately after plating the protoplasts without bursting protoplasts. High osmotic pressure impairs metabolism and growth of protoplasts (Evans and Bravo, 1983). This can cause reduced uptake of amino acids across the plasma membrane (Reusink, 1978) and reduced cell wall regeneration (Pearce and Cocking, 1973). Another consideration is that the agarose combined with higher concentration of sugar used even at the early culture did not play a favorable role on the protoplasts growth from the beginning culture, but the agarose combined with low concentration of sugar increase plating efficiency and colony formation. The agarose may increase protoplast stability by immobilizing protoplasts in agarose so that low osmotic condition of bead culture can be used immediately after plating without bursting protoplasts. Schnabl and Youngman (1985) reported that immobilization of protoplasts in alginate leads to an increased stability mainly of a mechanical nature, thus prolonging the life of the cells. A further possibility is that immobilization may tend to aid cell wall regeneration of the protoplasts.

Effects of hormone concentrations of liquid media on plating efficiency and colony formation in bead culture The plating efficiency and colony formation in A8 liquid medium were significantly higher than those in A7 and A9 liquid media (Table 1.3). Hormone concentrations lower or higher than that of A8, significantly decreased plating efficiency and colony formation.

Effects of types of culture on plating efficiency and colony formation The first cell division occurred 1 day after plating in bead culture and after 1 week in agarose layer and liquid cultures. Cells usually divided 1 week after plating in the bead culture and 3 weeks after plating in the other two culture types. The first colony formation appeared 3 days after plating in bead culture and after 3 weeks in the other two cultures. Colonies grew up to 0.48 mm diam. 1 week after plating in bead culture and 0.47 mm diam. after 4 weeks in the other two cultures. Cell division in bead culture was much more rapid than in the other two cultures.

Bead culture using solid KM6 and liquid A8 media led to a statistically significant increase in plating efficiency (19.1% vs. 1.8% in agarose layer culture and 1.3% in liquid culture) and colony formation (15.5% vs. 2.5% in agarose layer culture and 2.0% in liquid culture) (Table 1.4). There was no significant difference between agarose layer and liquid cultures for either plating efficiency or colony formation. In these experiments, the same callus-inducing medium, protoplast isolation procedure, and KM basal medium as those of Elmer et al. (1989) were used except different protoplast culture methods and cultivars or NAA for asparagus protoplast culture. The plating efficiency (19.1%) and colony formation (15.5%) counted 1 week from bead culture was

Table 1.3. Effects of hormone concentrations of liquid media on plating efficiency and colony formation in bead culture.

Liquid medium	Plating efficiency (%)[1] (cell divided / total protoplasts plated)	Colony formation (%)[1] (colonies > 3 cells / total protoplasts plated)
A 8	19.12 A	15.52 A
A 7	11.53 B	7.54 B
A 9	6.92 C	5.13 C

[1]Means of 6 replicate plates from all three isolations. Means within a column with different letters are significantly different (Duncan's multiple range test, $P < 0.05$). Plating efficiency and colony formation was evaluated 1 week after isolation.

Table 1.4. Effects of liquid, agarose layer and bead type cultures on plating efficiency and colony formation.

Type of culture	Plating efficiency (%)[1] (cells divided / total protoplasts plated)	Colony formation (%)[1] (colonies > 3 cells / total protoplasts plated)
Bead	19.12 A	15.52 A
Agarose layer	1.75 B	2.48 B
Liquid	1.31 B	1.96 B

[1]Means of 6 replicate plates from all three isolations. Plating efficiency and colony formation were determined 1 week after isolation for the bead culture; plating efficiency was evaluated 3 weeks and colony formation recorded 5 week after isolation for the agarose layer and liquid cultures. Means within a column with different letters are significantly different (Duncan's multiple range test, $P < 0.05$).

a significant improvement over previous asparagus protoplast regeneration procedures (Bui Dang Ha et al., 1975; Elmer et al., 1989) in which Elmer et al. reported a maximum plating efficiency of 7.3% obtained 3 weeks after plating. Kong et al. (1988) reported that a maximum about 10% of the cells were divided 20 days after plating using agarose on polypropylene membrane, and sizable colonies formed about 40 days after growing colonies on the membrane in asparagus protoplast culture.

Bead culture was very effective in promoting protoplast division in this study, similar to observations made by others (Shillito et al., 1983; Thompson et al., 1986; Furze et al., 1987). The bead culture technique combines the advantages of high and low density culture. The protoplasts can be plated close together to maximize conditioning effects, while a large pool of medium is available for continued growth. Moreover, the large volume of liquid culture medium dilutes substances released by the developing cells that may be inhibitory or toxic to the protoplasts (Shillito et al., 1983).

Comparison of different media on shoot induction Microcalli grew from 0.5 - 1.0 mm diam. initial to as large as 6 mm diam. within 1 week after transfer from bead culture to shoot-inducing media (Figure 1.2b). Shoot primordia (light yellow structures, about 1 to 3 mm diam. and 3 mm to 1 cm long) were observed on calli cultured in shoot-inducing media only with 0.25 mg l^{-1} kinetin and 0.25 mg l^{-1} or 1 mg l^{-1} 6-BAP. Four weeks later, calli were transferred into hormone free MS medium. Green shoot primordia (1 to 5 mm diam. and 3 mm to 1 cm long) were found on calli cultured on all shoot-inducing media and the control medium following transferral to hormone-free medium. Shoots developed within 1 week to 3 months on the hormone-free medium only

after transferral from the shoot-inducing media (Figure 1.2c). Shoot growth did not occur on calli cultured on control medium or on calli maintained permanently on media containing hormones. These results showed that the hormones used in shoot-inducing media were necessary for induction of the shoots, but inhibited further shoot development.

The most effetive shoot-inducing medium contained 0.25 mg l^{-1} 6-BAP combined with 0.125 mg l^{-1} each of NAA and 2,4-D and resulted in an average shoot regeneration of 92.3% (Figure 1.3). The combination of 6-BAP and auxins was also the most effective in stimulating the regeneration of shoots from the calli (Figure 1.3). Shoot formation was not observed after calli were transferred from media with 1 mg l^{-1} kinetin combined with 0.25 mg l^{-1} each of NAA and 2,4-D or 1 mg l^{-1} kinetin without auxins and 0.25 mg l^{-1} zeatin and 0.125 mg l^{-1} each of NAA and 2,4-D.

Shoots developed directly into plants after calli were transferred from shoot-inducing media onto hormone-free medium (Figure 1.2d). The plant regeneration was 14%, 16%, 38% and 79% of total shoots obtained from each shoot-inducing medium respectively with 0.25 mg^{-1} 6-BAP + 0.125 mg^{-1} NAA + 0.125 mg^{-1} 2,4-D; 0.25 mg^{-1} kinetin + 0.125 mg^{-1} NAA + 0.125 mg^{-1} 2,4-D; 1 mg^{-1} 6-BAP + 0.25 mg^{-1} NAA + 0.25 mg^{-1} 2,4-D and 1 mg^{-1} 6-BAP. After plants were transferred into the greenhouse, 71% of them survived (Figure 1.4).

CONCLUSION

Critical factors for effective protoplast regeneration were protoplast culture medium, types of culture, osmotic condition and hormone concentrations of liquid media in bead

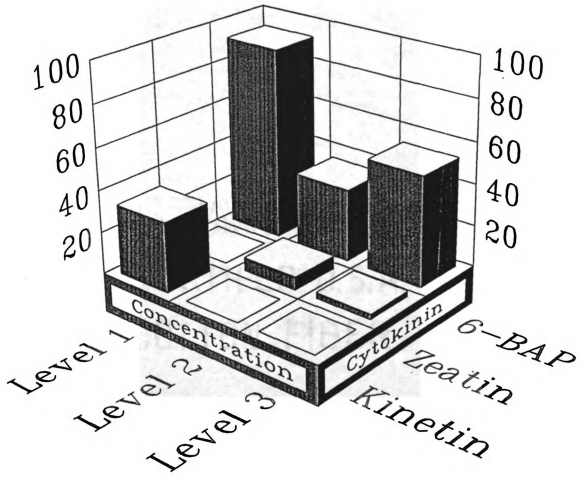

Figure 1.3. Effect of cytokinin type and concentration, in combination with auxins in the shoot-inducing medium of protoplast-derived calli of asparagus, on shoot regeneration (%). Level 1: 0.25 mg l^{-1} cytokinin with 0.125 mg l^{-1} each of NAA and 2,4-D. Level 2: 1 mg l^{-1} cytokinin with 0.25 mg l^{-1} each of NAA and 2,4-D. Level 3: 1 mg l^{-1} cytokinin alone. Data represent the mean of 3 replicate experiments.

Figure 1.4. Asparagus plants regenerated from protoplasts growing in the greenhouse.

culture and shoot-inducing medium. The high plating efficiency, colony formation and plant regeneration achieved in this investigation offers an opportunity to develop *Fusarium* resistant asparagus cultivar through selection of resistant somaclonal variation, genetic transformation and protoplast fusion.

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CHAPTER II

The Development of *Asparagus officinalis* cv. Lucullus 234 Somaclones with High levels of Resistance to *Fusarium* spp.

ABSTRACT

Asparagus somaclones were produced by subculturing shoots initially generated from callus-derived protoplasts of *A. officinalis* cv. Lucullus 234 through callus cycles for over a three year period. One hundred and twenty protoplast-derived somaclones of 'Lucullus 234' were screened for resistance to two virulent Michigan isolates each of *Fusarium oxysporum* f. sp. *asparagi* Cohen and Heald (FOA10 and FOA50) and *F. proliferatum* (T. Matsushima) Nirenberg (FM12 and FM49) in the greenhouse. Somaclones had significantly increased levels of resistance overall to both *Fusarium* spp. over the vegetatively micropropagated 'Lucullus 234' parental plants. After somaclones containing higher levels of resistance (at rating scale 1 or 2) were vegetatively micropropagated, a minimum of 12 micropropagated plants of each were rescreened for resistance to the most virulent isolate FOA50 in the greenhouse. Two somaclonal lines, R7 and R4, were rated as highly significantly resistant to FOA50 in comparison with the vegetatively micropropagated parental plants. These lines were very similar horticulturally to the parental cultivar. Cytogenetic studies showed that R7 and R4 were

diploid with normal chromosomal number (2n=20) as 'Lucullus 234' parental cultivar (2n=20). Genomic fingerprints by randomly amplified polymorphic DNAs (RAPDs) showed that the resistant somaclonal lines R7 and R4 may have carried DNA sequence mutations as their DNA sequences varied from the 'Lucullus 234' parental plant.

INTRODUCTION

Since the first successful methods for in vitro growth of carrot (Gautheret, 1939; Nobecourt, 1939) and tobacco (White, 1939) tissues were reported in 1939, tissue-culture techniques had been subsequently developed and refined for hundreds of plant species. Two observations noted during preliminary studies of cultured cells and regenerated plants in vitro are that: 1) genetic and cytogenetic variation commonly increases during exposure of cells to in vitro growth, and 2) while much phenomenology has been recorded, the mechanisms generating the variation remain to be described. Early variant plants regenerated from cell cultures of geranium were termed "calli-clones" by Skirvin and Janick (1976a), while plants regenerated from protoplasts of potato were termed "protoclones" by Shepard *et al.* (1980). It was finally in 1981 that Larkin and Scowcroft named the genetic variability in plants regenerated from tissue and cell culture as "somaclonal variation", a term which has been widely accepted (Larkin and Scowcroft, 1981).

Somaclonal variation comes from: 1) preexisting differences in individual somatic cells revealed by enabling these to regenerate in differentiated plants by tissue culture and 2) culture-induced changes (Bajaj, 1990). Somaclonal variation has resulted in epigenetic variation and genetic variation. Epigenetic changes are usually induced by tissue culture

condition and are not expressed in the sexual progeny of regenerated plants. Genetic changes have been detected either in spontaneously generated mutants or as a result of induced mutations (Evans, 1989). A earlier major achievement in this direction was made by Skirvin and Janick (1976a), although their work did not receive much attention at that time. They recovered variant plants differing in their oil component, fasicination, pubescence, and anthocyanin production from callus cultures of scented geranium.

Extensive studies conducted during the last decade have shown that the cell cultures, especially in long term cell cultures, undergo various morphological and genetic changes including polyploidy, aneuploidy, chromosome breakage, deletion, translocation, gene amplification, inversion, single gene mutation, etc. (Meins, 1983; D'Amato, 1985). In the early 1980s, Pelletier and Pelletier (1971) had found such culture-induced variations including chlorophyll deficiency and enhanced axillary shooting in lettuce cultures. Interestingly, in all plants which showed such variations the chromosomal number was normal. Further genetic-based information from anther culture-induced variations only recognized ploidy changes and chromosomal number responsible for such variations (Devreux and Laneri, 1974; Collin and Legg, 1980; Oono, 1981). However, Alhoowalia (1976 and 1978) for the first time reported gross structural changes including reciprocal, translocation, deletion and inversions responsible for the production of variants in *Lolium*. In addition, the molecular basis of somaclonal variation has been studied. The detected change of somaclonal variant in maize was a single base pair alteration that resulted in a change from glutamic acid to valine in the last codon of exon 6 (Brettell *et al.*, 1986). Nuclear ribosomal DNA spacer length variation has been found in somaclonal variants of a spring wheat (Rode *et al.*, 1987). Grossly altered methylation

pattern, which resulted in epigenetic variation, have been reported in somaclonal variants (Brown and Lorz, 1986). Qualitative and quantitative changes in gliadin proteins have been detected in somaclonal variants of wheat (Maddock *et al.*, 1985; Cooper *et al.*, 1986). Organelle DNA changes were found in somaclonal variants of potato (Kemble and Shepard, 1984).

The first work recognizing the potential of somaclonal variation as a source of variability for crop improvement was conducted with sugarcane (Heinz and Mee, 1969) and later with potatoes (Heinz, 1973; Nickell, 1977; Shepard *et al.*, 1980). These workers found that plants regenerated from callus and protoplast cultures varied in morphological characteristics, maturity date, yield, and response to pathogens. Since then tremendous progress in this area have been made. Agriculturally useful somaclonal variants have been identified which have desirable traits such as increased solids in tomato, male sterility in tomato and rice, higher yields and enhanced protein production in rice, earliness in maize, freezing tolerance in wheat, and increased sugar content in sugarcane (Evans, 1989; Bajaj, 1990). Leaf, flower and color variants have been used to develop new breeding lines of ornamentals (Evans and Bravo, 1986).

Development of resistance to fungal, bacterial and viral diseases in various crops has been the major contribution of somaclonal variation (Lal and Lal, 1990). In alfalfa, somaclones resistant to *Fusarium oxysporum* were regenerated from cell lines selected for resistance to *Fusarium* culture filtrates (Hartman *et al.*, 1984). Screening of alfalfa somaclones regenerated from protoplasts resulted in plants that were resistant to *Verticillium albotrum* (Latunde and Lucas, 1983). Two somaclonal lines of rice were resistant to sheath blight in the field (TCCP, 1987). Daub (1986) has reported

protoplast-derived somaclones of tobacco cultivars with increased resistance to several major tobacco pathogens including TMV, *Meloidogyne incognita*, and *Phytophthora parasitica* var. *nicotianae*. She also identified three somaclones whose progeny enhanced resistance to black shank in both field and greenhouse. Resistance in celery to *Fusarium oxysporum* f. sp. *apii* race 2 has been enhanced with somaclonal variation (Toth and Lacy, 1991). Somaclonally-derived resistance to tomato mosaic virus and tobacco mosaic virus has been obtained in tomato (Smith and Murakishi, 1993). To date, six cultivars have been released that were derived from somaclonal variation. The sugarcane cv. Ono, which is higher yielding and shows increased resistance to Fiji disease, was developed by isolating subclones of sugarcane resistant to Fiji disease from a susceptible cultivar (Kirshnamurthi and Tlaskal, 1974). Velvet Rose, a scented geranium cultivar, has been developed by Skirvin and Janick (1976b). The sweet potato cultivar Scarlet, isolated from meristem tip culture-derived clones, has a desired quality trait of darker and more stable skin color (Moyer and Collins, 1983). DNAP 9 and DNAP 17, two tomato cultivars, have been shown to have higher solids in several field trails and resistance to *Fusarium* Race 2, respectively. The two cultivars were derived from plants regenerated from leaf explants (Evans, 1987). Bell sweet, a yellow bell pepper cultivar, was identified as a variant among plants regenerated from anther culture and was shown to have few or no seeds (Morrison and Evans, 1988).

To identify possible sources of resistance to *Fusarium oxysporum* and *F. proliferatum*, ninety five cultivars and breeding lines of four asparagus species, *Asparagus officinalis*, *A. densiflorus*, *A. setaceus* and *A. acutifolius*, were evaluated in response to one virulent Michigan isolate each of *F. oxysporum* (FOA10) and *F.*

proliferatum (FM12) in the greenhouse tests. A cultivar of *A. officinalis*, Lucullus 234, had the highest resistance to FOA10 and FM12 among 90 cultivars and breeding lines of this species tested. Two cultivars of an ornamental asparagus species, *A. densiflorus* 'Sprengeri' and 'Myersii' were more resistant than any other asparagus cultivar or species tested (Stephens *et al.*, 1989). However, sexual crosses of *A. officinalis* with the resistant species, *A. densiflorus*, have been unsuccessful, probably due to incompatibility barriers (Elmer *et al.*, 1989).

A previous study by Dr. Smither in this laboratory screened somaclones of 2 cultivar of *A. officinalis*, 'Lucullus 234' and 'Jersey Giant'. Somaclones of 'Lucullus 234' exhibited a higher level of resistance to *F. oxysporum* and *F. proliferatum* than the 'Jersey Giant' somaclones. In addition, somaclones of 'Lucullus 234' were more resistant to *F. proliferatum* than *F. oxysporum* (M.L. Smither and C.T. Stephens, unpublished observation).

The genome provides a source of constant primary character, and the cultivar identification, measuring levels of genetic diversity, genetic mapping, tagging genes of interest in many cultivated plants have been done by using randomly amplified polymorphic DNAs (RAPDs) analysis (O'Brien, 1990; Quiros *et al.*, 1991). Although the genetic bases of somaclonal variations in other crops have been determined by the techniques described above, interest in RAPDs is rapidly growing because their identification is simple and fast. In addition, their polymorphisms among the amplification products are detected frequently and through examination of an ethidium bromide-stained agarose gel instead of using radioactivity (Williams *et al.*, 1990). The RAPD PCR technique has been developed by Williams *et al.* (1990). This technique

relies on the differential enzymatic amplification of random DNA fragments using PCR with single primers of arbitrary nucleotide sequence (usually 10-mers), but require no knowledge of target DNA sequence. Polymorphisms result from either chromosomal changes in the amplified regions or base changes that alter primer binding. The RAPD markers are usually dominant because polymorphisms are detected as the presence or absence of bands. RAPD markers can be used for genetic mapping, identification of the genomic regions derived from the trait donor parent of near-isogenic lines and F_2 pool selection, study of population genetics and molecular taxonomy (Williams *et al.*, 1993). The RAPD markers linked to disease resistant genes have been successfully identified in tomato (Martin *et al.*, 1991) and lettuce (Michelmore *et al.*, 1991). The RAPD PCR was also used to identify grapevine cultivars (Collins and Symons, 1993).

The goal of this study was to select protoplast-derived somaclones of 'Lucullus 234' with higher levels of resistance to two Michigan virulent isolates each of *Fusarium oxysporum* (FOA10 and FOA50) and *F. proliferatum* (FM12 and FM49) in the greenhouse, test the stability of the resistance of selected individual more resistant somaclones, and finally characterize the genetic basis of the resistant somaclones. In this investigation, we report: 1) development of a protocol for the production of protoplast-derived somaclones , 2) development of a protocol for vegetative micropropagation of 'Lucullus 234' seed plants, 3) the screening of 120 protoplast-derived somaclones of 'Lucullus 234' for resistance to FOA10, FOA50, FM12 and FM49 in the greenhouse, 4) the rescreening of a minimum of 12 vegetatively micropropagated plants of each more resistant somaclone in response to FOA50 in the greenhouse, 5) a preliminary characterization of the genetic basis of the resistant somaclones using the RAPD PCR and

chromosome counts.

MATERIALS AND METHODS

Production of somaclones Due to the higher *Fusarium* resistance of 'Lucullus 234' previously reported (Stephens *et al.*, 1989) and the highest probability to get variants from plants regenerated from protoplasts compared with other types of culture such as callus, stems, somatic embryos, adventitious buds and etc. (Demarly, 1986), initial shoots regenerated from callus-derived protoplasts of *A. officinalis* cv. Lucullus 234 (Dan and Stephens, 1991) were used to produce somaclones. These shoots were cultured on Murashige & Skoog (MS) medium (1962) supplemented with 0.1 mg/l 6-BAP (RM17), calli formed on the shoots within 15 to 30 days, and new shoots developed on the calli within 30 days. By using this technique, shoot production was maintained for over 3 years and the produced shoots were used as the somaclonal source because accumulation of variability has been positively correlated with duration of culture (Evans *et al.*, 1984). Effects of different root inducing agents: auxins (NAA, 2,4-D, IAA and IBA), ancymidol and sucrose at different concentrations and combinations on root differentiation of the produced shoots were examined. Rooted plants were cultured in a hormone-free MS medium for shoot and root elongation for another month. All cultures were placed at 27° C with 40 $\mu Em^{-2}s^{-1}$ light and a 16 h photoperiod.

Before plants were transferred to the greenhouse, they were acclimatized in order to adapt to greenhouse conditions. Plants were transferred to pots containing synthetic media (Baccto, Michigan Peat Co., Houston, Texas) (1 vermiculite : 1 Peat : 1 Perlite).

The pots were placed in a plastic bag at 22 to 24° C under cool-white fluorescent light and a 24 hour photoperiod. The bags were completely sealed for the first week, 3 holes punctured in the sealed bag for the second week, and opened for the third week. The pots were maintained one additional week after the plastic bag was completely removed at the end of the third week, and then were moved to the greenhouse.

Vegetative micropropagation of 'Lucullus 234' Due to limited number of 'Lucullus 234' seeds, it was necessary to develop a protocol to vegetatively micropropagate 'Lucullus 234' plants. Two different concentrations of cytokinin 6-BAP were tested for shoot production. Spears of greenhouse grown 'Lucullus 234' were surface sterilized (Chapter I) and cut into single-node segments. Two to four segments were cultured on 20 ml of either one of two different modified MS media supplemented with 0.5 mg/l (RM18) and 0.1 mg/l (RM17) of BAP in 25 X 150 mm culture tubes. The effects of four different auxins IAA, IBA, NAA, and 2,4-D on root induction were tested. Branched shoots at the node region were excised after 15 day culture in RM17 medium. Two shoots were cultured in a culture tube having 20 ml each of four different root-inducing media containing basic MS medium, and IAA, IBA, NAA, and 2,4-D, respectively, each at concentration of 2 mg/l. IBA was found to result in the highest root production. Further, five concentrations of IBA, 3 mg/l, 5 mg/l, 7 mg/l, 9 mg/l and 11 mg/l, were tested for improving rooting efficiency. Rooted plants were cultured on a hormone-free MS medium for further shoot and root development for one month. All cultures were maintained at 27° C with 40 $\mu Em^{-2}s^{-1}$ light and a 16 h photoperiod. Plants were transferred into the greenhouse after acclimazation as described above. All

experiments contained at least 8 replicates per treatment and were repeated 4 times.

The first cycle of screening for higher levels of *Fusarium* resistance in somaclones in the greenhouse One hundred and twenty somaclones and 120 vegetatively micropropagated plants of 'Lucullus 234' generated as described above were used for screening to the *Fusarium isolates*. Previous work demonstrated that a cultivar of *A. officinalis*, 'UC 157', is highly susceptible to *F. oxysporum* and *F. proliferatum*, and a cultivar of an ornamental asparagus species *A. sprengeri*, 'Sprengeri', is highly resistant (Stephens *et al.*, 1989). 'UC 157' and 'Sprengeri' were used as susceptible and resistant controls, respectively, for the screening test.

Seeds of *Asparagus* spp. were surface disinfected according to the method of Stephens *et al.* (1988), and germinated in agar medium (1.5%, w/v) at room temperature. One week later, germinated seeds were transferred into pots containing synthetic medium as described above and grown in the greenhouse. Inocula of two Michigan virulent isolates each of *F. oxysporum* (FOA10 and FOA50) and *F. proliferatum* (FM12 and FM49) were prepared using the method of Wacker *et al.* (1990). Each *Fusarium* inoculated and *Fusarium*-free soils (control soils) were amended with soil mix. One-month-old seedlings of 'UC 157' and 'Sprengeri', and one-month-old-greenhouse grown somaclones and vegetatively micropropagated plants of 'Lucullus 234' were transferred to 53 x 28 x 6 cm tray containing 7 kg of *Fusarium* infested soil. Plants were fertilized (Peters 20N-20P-20K, 200 ppm) once per week.

Plants were harvested after 1 to 4 months depending on weather conditions such as temperature or humility. Disease incidence was assessed using a visual rating scale

(Stephens *et al.*, 1989; Wacker *et al.*, 1990): 1= healthy plant, no evidence of disease (highly resistant), 2= few root lesions, <25% diseased (resistant), 3= moderate number of root lesions, <50% (moderately susceptible), 4= many root lesions, <75% (susceptible), and 5= all roots flaccid or dead (highly susceptible). Experiments were designed as a two factorial experiment using randomized complete block design for *Fusarium* spp. as factor A and germplasm entries as factor B , which is a split plot on A. All experiments were arranged on greenhouse benches under a natural photoperiod at 25 to 30°C. Data were analyzed by MSTAT program for ANOVA.

Test stability of the *Fusarium* resistance in the more resistant somaclones selected from the first cycle To determine if *Fusarium* resistance in the individual somaclones with higher levels of resistance selected from the first cycle remain stable, somaclones with a disease rating of 1 and 2 were maintained in the greenhouse for further disease screening. Each of the selected somaclones was vegetatively micropropagated using the method described above except RTM30 medium was used for root induction. At least twelve micropropagated plants of each of the more resistant somaclones were rescreened for resistance to FOA50 as described above. FOA50 was chosen as the screening agent in these tests because FOA50 was found to be more virulent to 'Lucullus 234' and the controls. All experiments were conducted as a randomized block design in the greenhouse under the same conditions as described above. Data were subject to Student's test.

Cytogenetic study of the resistant somaclones In order to examine changes of ploidy of the somaclones with increased resistance (R4 and R7), their root tips were treated in 0.2% 1-bromonaphthaline for 3 hours and fixed in acetic acid: alcohol (1:3) for 24 hours after rinsing in running tap water. They were rinsed in distilled water, macerated in 1 N HCl at 60°C for 15 min, and stained in acetocarmine (1%, w/v) for 24 hours then squashed for observation of chromosomes under a compound microscope. Pictures of chromosome counts were taken under a laser scanning confocal microscope (LSM) Model 210 (Carl Zeiss, Inc., Thornwood, N.Y.) with a Matrix Multicolor computerized camera unit (Agfa-Matrix, Orangeburg, N.Y.) which receives signals directly from the LSM.

Determining the genetic bases of the resistant somaclones by RAPDs The more resistant somaclonal lines R7 and R4 selected from the second cycle of screening and four randomly chosen seed plants of 'Lucullus 234' (controls) were screened for RAPDs by using 20 arbitrary primers to preliminarily measure genetic variability in the resistant somaclones.

Genomic DNA was isolated from shoots of greenhouse grown plants using the protocol of Dellaporta *et al.* (1985). The isolated DNA was then purified according to the method of Fang *et al.* (1992) to remove polysaccharides which inhibit the activity of many molecular biological enzymes, such as restriction enzymes and Taq polymerase (Fang *et al.*, 1992). Two separate DNA isolations were made, to document the reproducibility of the amplification products.

PCR was carried out with a 110S Programmable Coy TempCycler II (COY corporation, Gass Lake MI), 20 arbitrary primers (Table 2.1) (Operon Technologies,

Table 2.1. Source of twenty primers used to screen the resistant somaclones R7, R4 and four control plants.

Primer	Sequence	Primer	Sequence
A	CCTACACGGT	K	ACTGGGCCTC
B	CAGCCGAGAA	L	GACGCAGCTT
C	GAAGGAGGCA	M	CCGAGGTGAC
D	TTGCGGCTGA	N	GGTGCGCACT
E	CCCGATCAGA	O	CACGAACCTC
F	CCGCAGTCTG	P	TCCCGGTGAG
G	GGAAAGCGTC	Q	TGAACCGAGG
H	CTCTGCCTGA	R	GTGTCCCTCT
I	CCCCTCAGAA	S	GGACAAGCAG
J	GGTTGGAGAC	T	CTCCGCACAG

Alameda, CA) and GeneAmp PCR Core Reagents (Perkin Elmer Cetus, Norwalk CT). To determine the optimal concentration of $MgCl_2$ for PCR of asparagus genomic DNA, 1.5 to 5 mM of $MgCl_2$ at 0.5 mM interval was used. The concentration of 1.5 mM of $MgCl_2$ was chosen for PCR because it was found to be the only concentration which provided PCR products. PCR reaction mixtures contained 25 μl of 1X PCR buffer (500 mM KCl, 100 mM Tris-HCl, pH 8.3), 200 μM each of dATP, dCTP, dGTP, and dTTP, 0.4 μM primer, 33 ng/μl T4 gene 32 protein (Pharmacia Biotech Inc. Piscataway NJ), 0.04 U/μl AmpliTaq DNA polymerase, 1.5 mM $MgCl_2$ and 2 ng/μl genomic DNA, and the reaction mixture overlaid with 25 μl of sterile mineral oil (Sigma Chemical Co., St. Louis, MO). The PCR program consisted of an initial denaturation of 7 min at 94°C, 45 cycles of 1 min at 94°C, 1 min at 35°C, 2 min at 72°C, and a final extension step of 15 min at 72°C. The PCR products were size-separated by electrophoresis in 1.4% agarose (Sigma Chemical Co., St. Louis, MO) in 1X TAE at 2.5 V/cm in a cold room (4°C) for 24 hours.

RESULTS AND DISCUSSION

Production of somaclones Initial shoots generated from callus-derived protoplasts of *A. officinalis* (Dan and Stephens, 1991) were cultured on shoot-inducing medium RM17. Fifteen to 30 days later, calli were produced on the shoots, and new shoots developed on the calli within 30 days. Shoot production was maintained for over 3 years using this procedure. When the produced shoots were transferred into different root-inducing media, shoots formed a small compact mass resembling a crown where the thick roots

were attached, and then roots produced from the mass. The highest average root production (55.5%) was obtained from shoots cultured four weeks in a MS medium containing IBA at 11 mg l^{-1} (data not shown). Further root development was much quicker when shoots with induced roots were cultured in a hormone-free MS medium as opposed to shoots cultured in initial root-induction media. This indicated that IBA, which is necessary for root induction, inhibited further root growth. Rooting of shoots regenerated from protoplasts in this study was not improved by ancymidol or by increasing the concentrations of sucrose in contrast to the results reported by Chin (1982) and Desjardins *et al.* (1987) (data not shown). Two hundred plants were transferred into the greenhouse, 80.5 % of them survived and were used as a source of somaclones.

Vegetative micropropagation of 'Lucullus 234' Shoots (1 to 2 cm in length) developed from more than 90% of nodes of spears 15 days after single-node segments were cultured on RM17 medium. Roots appeared usually 15 to 30 days after the produced shoots were cultured in root-inducing media. Among four different auxins tested, IBA produced the highest rooting of 33.3% in one month (Figure 2.1). The auxin, 2,4-D, completely inhibited root induction (Figure 2.1). The highest average of 57.5% of shoots having roots was obtained 1 month after the shoots were cultured in RTM 26 containing MS medium + IBA 3 mg/l (Figure 2.2). One month after culture in RTM26, all the plantlets possessed well-developed crowns with 4 to 7 spears and vigorous roots (Figure 2.3). At this stage, plants were transferred to a hormone-free MS medium for further shoot and root development for one month. Two hundred and fifty seven plants were transferred to the greenhouse after acclimazation as described above, and 84.4% of them

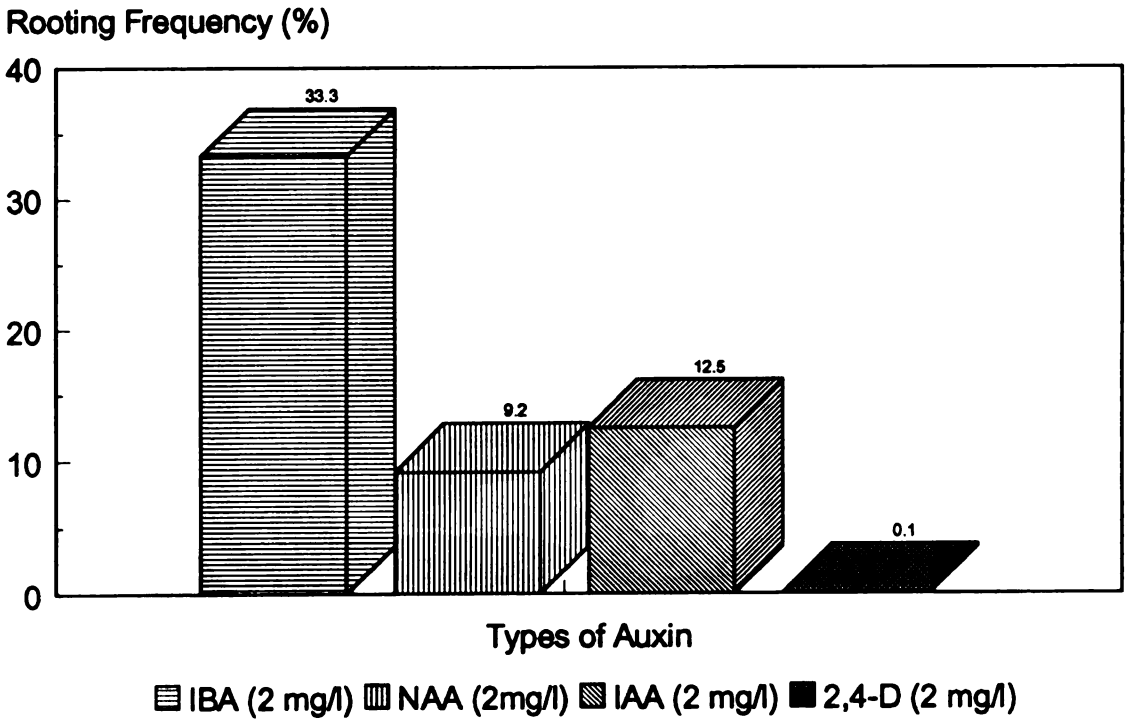

Figure 2.1. Effects of different auxins (IAA, IBA, NAA, and 2,4-D) on rooting frequency (%) of the shoots produced on RM17 medium of asparagus cv. Lucullus 234 after 4 weeks in culture.

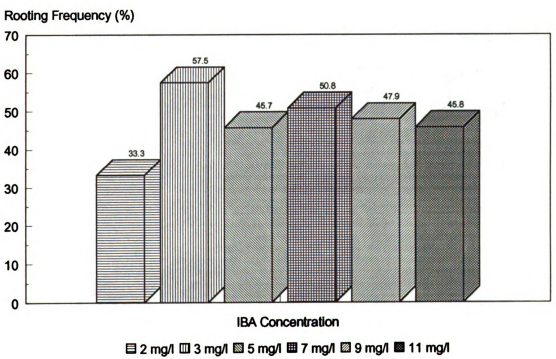

Figure 2.2. Effects of IBA concentrations on rooting frequency (%) of the shoots produced on RM17 medium of asparagus cv. Lucullus 234 after 4 weeks in culture.

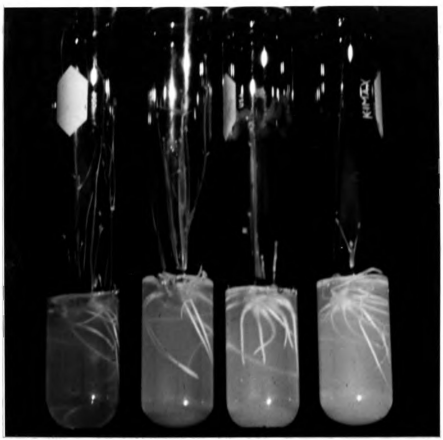

Figure 2.3. Four-week-old plantlets of asparagus cv. Lucullus 234 in RTM26 medium containing basic MS medium + 3 mg/l IBA.

survived.

Screening of somaclones for *Fusarium* resistance For the first cycle of screening somaclones for *Fusarium* resistance, 120 somaclones of 'Lucullus 234', and 120 plants of vegetatively micropropagated 'Lucullus 234' parental plants, 'Sprengeri' and 'UC 157' were screened for resistance to FOA10, FOA50, FM12 and FM49. Both different *Fusarium* spp. and germplasm had a significant effect on *Fusarium* disease incidence (Table 2.2). The interaction between germplasm and *Fusarium* spp. did not significantly affect *Fusarium* disease incidence (Table 2.2). FOA50 was significantly more virulent to all plants when compared to FOA10 (data not shown). Somaclones were significantly more resistant to both *Fusarium* spp. when compared to the vegetatively micropropagated 'Lucullus 234' parental plants (Table 2.3). Of 120 somaclones, 24.2% of them (vs. 8.3% of the parental plants) fell into rating scale 1, 40.8% (vs. 41.6% of the parental plants) in rating scale 2, 15.8% (vs. 25.8% of the parental plants) in rating scale 3, 8.3% (vs. 10.8% of the parental plants) in rating scale 4, and 10.8% (vs. 13.3% of the parental plants) in rating scale 5 (Table 2.4). Of 120 plants of 'Sprengeri', 50.8% of them fell into rating scale 1, 47.5% in rating scale 2, 1.6% in rating scale 3 and none in rating scale 4 and 5. Of 120 plants of 'UC 157', none of them fell into rating scale 1, 17.5% in rating scale 2, 40.8% in rating scale 3, 27.5% in rating scale 4 and 14.2% in rating scale 5 (Table 2.4). Ninety two percent of the somaclones and vegetatively micropropagated plants of 'Lucullus 234', and 'Sprengeri' remained healthy when they were planted in control soils amended with soil mix without the *Fusarium* isolates, and 75% of 'UC 157' were healthy. The rest of all the plants had a few root lesions (at

Table 2.2. Analysis of variance of a randomized complete block design for *Fusarium* spp. as factor A and germplasm entries as factor B, which is a split plot on A.

Source	Degrees of freedom	Mean square	F-test[x]
Block	5	0.522	NS
Factor A (*Fusarium* spp.)	3	1.658	S
Error	15	0.353	
Factor B (Germplasm)	3	14.840	S
AB	9	0.533	NS
Error	60	0.384	
Total	95		

[x]NS = F-test not significant.

S = F-test significant ($P<0.05$).

Table 2.3. Mean disease rating of asparagus plants screened with *Fusarium oxysporum* (isolates FOA10 and FOA50) and *F. proliferatum* (isolates FM12 and FM 49) in the greenhouse.

Plant source	Treatments[x]				Total mean disease rating[y]
	FM12	FM49	FOA10	FOA50	
'UC157' (susceptible control)	3.47	3.1	3.00	3.97	3.38 A
Micropropagated seed plants of 'Lucullus 234'	2.77	2.50	2.67	3.23	2.79 B
Somaclones of 'Lucullus 234'	2.23	2.90	1.80	2.70	2.41 C
'Sprengeri' (resistant control)	1.63	1.47	1.40	1.53	1.51 D

[x]One hundred and twenty plants of each plant sources were screened at four different times. Disease was assessed using a visual rating scale: 1= no disease, 2= < 25% diseased, 3= < 50% diseased, 4= < 75% diseased, and 5= > 75% diseased.

[y]Means disease rating of four treatments (FM12, FM49, FOA10 and FOA50). Means within a column with different letters are significantly different (Duncan's multiple range test, $P < 0.05$).

Table 2.4 Disease rating of asparagus plants screened with *Fusarium oxysporum* (isolates FOA10 and FOA50) and *F. proliferatum* (isolates FM12 and FM49) in the greenhouse.

Plant source	No. of plants in disease rating scales[x]					Mean disease rating[y]
	1	2	3	4	5	
'UC157' (Susceptible control)	0	21	49	33	17	3.38 A
Micropropagated seed plants of 'Lucullus 234'	10	50	31	13	16	2.74 B
Somaclones of 'Lucullus 234'	29	49	19	10	13	2.41 C
'Sprengeri' (Resistant control)	61	57	2	0	0	1.51 D

[x]One hundred and twenty plants of each of plant source were screened at four different times. Disease was assessed using a visual rating scale: 1= no disease, 2= < 25% diseased, 3= < 50% diseased, 4= <75% diseased, 5= >75% diseased.

[y]Means of disease rating within a column with different letters are significantly different (Duncan's multiple range test, $P < 0.05$).

rating scale 2) when they were planted in the control soil (data not shown).

To determine stability of the *Fusarium* resistance in the most resistant somaclones selected from the first cycle, 54 somaclones with a disease rating of 2 or less were maintained in the greenhouse for further disease screening. Of 54 somaclones maintained in the greenhouse, 15 (27.7%) of them developed spears which could be used as explants for micropropagation. The 15 somaclones were then vegetatively micropropagated as described above. Only two somaclones (R7 and R4) could develop healthy roots in vitro. At least 12 micropropagated plants of each of R7 and R4 were rescreened with FOA50 in the greenhouse using a randomized complete block design. Both somaclonal lines R7 and R4 were highly significantly resistant to FOA50 in comparison with the vegetatively micropropagated 'Lucullus 234' parental plants (Table 2.5 and 2.6). Of 12 micropropagated plants of R7, 91.7% of them were rated at 1 or 2 in contrast to 6.67% of the parental plants (Table 2.5). Of 18 micropropagated plants of R4, 50% of them were rated at 1 or 2 in contrast to 6.7% of the parental plants (Table 2.6). These results indicate that the increased levels of resistance of R7 and R4 to the *Fusarium* isolates from the first cycle of screening is stable after vegetative micropropagation. In addition, these lines were very similar horticulturally to the parental cultivar.

FOA10 had significantly lower virulence to all plants tested when compared to FOA50. Although 'Lucullus 234' was the most resistant to FOA10 and FM12 in comparison with 90 cultivars and breeding lines of this species tested (Stephens *et al.*, 1989), our results showed that the vegetatively micropropagated plants of 'Lucullus 234' seed plants were as susceptible to FOA50 as 'UC 157' (Table 2.5 and 2.6)

Asparagus somaclones of 'Lucullus 234' expressed a higher level of resistance to

Table 2.5. Disease rating of vegetatively micropropagated plants of the more resistant somaclone R7 rescreened with *Fusarium oxysporum* (isolate FOA50) in the greenhouse.

Plant source	No. of plants in disease rating scales[x]					Mean disease rating[y]
	1	2	3	4	5	
'UC157' (susceptible control)	0	1	9	2	3	3.47 A
Micropropagated seed plants of 'Lucullus 234'	0	1	12	1	1	3.13 A
Micropropagated plants of somaclone R7 of 'Lucullus 234'	3	8	1	0	0	1.83 B
'Sprengeri' (resistant control)	14	1	0	0	0	1.07 C

[x]Twelve vegetatively micropropagated plants of R7 were screened with FOA50. Disease was assessed using a visual rating scale: 1= no disease, 2= < 25% diseased, 3= < 50% diseased, 4= < 75% diseased, and 5= > 75% diseased.

[y]Means within a column with different letters are significantly different (Student's *t* test, $P < 0.01$).

Table 2.6. Disease rating of vegetatively micropropagated plants of the more resistant somaclone R4 rescreened with *Fusarium oxysporum* (isolate FOA50) in the greenhouse.

Plant source	No. of plants in disease rating scales[x]					Mean disease rating[y]
	1	2	3	4	5	
'UC157' (susceptible control)	0	1	5	5	4	3.80 A
Micropropagated seed plants of 'Lucullus 234'	0	1	7	4	3	3.60 A
Micropropagated plants of Somaclone R4 of 'Lucullus 234'	1	8	6	2	1	2.67 B
'Sprengeri' (resistant control)	14	1	0	0	0	1.07 C

[x]Eighteen vegetatively micropropagated plants of R4 were screened with FOA50. Disease was assessed using a visual rating scale: 1= no disease, 2= < 25% diseased, 3= < 50% diseased, 4= < 75% diseased, and 5= > 75% diseased.

[y]Means within a column with different letters are significantly different (Student's *t* test, $P < 0.01$).

the *Fusarium* isolates than the vegetatively micropropagated 'Lucullus 234' parental plants after the first cycle of screening. The vegetatively micropropagated plants of 2 somaclones with higher resistant levels were rescreened to the most virulent isolate FOA50. These two somaclonal lines, R7 and R4, were rated as highly significantly resistant in comparison with the parental plants, indicating the maintenance of the increased resistance in R7 and R4 lines after vegetative micropropagation. Highly resistant somaclones were regenerated at a high frequency of 24.2% (vs. 8.3% in the parental plants of 'Lucullus 234') from the moderately resistant cultivar Lucullus 234 after the first cycle of screening. Ninety two percent of the vegetatively micropropagated plants of R7 had disease ratings of 1 or 2, whereas 93.3% of the parental plants had rating of 3 or greater. Fifty percent of the vegetatively micropropagated plants of R4 had rating of 1 or 2, while 93.3% of the parental plants had rating of 3 or more than 3.

Measuring genetic variability in the resistant somaclones In order to understand the origin of the resistant somaclones, R7 and R4 were first cytogenetically studied using chromosome counts. Both somaclonal lines showed diploid with normal chromosome numbers (2n = 20) as their 'Lucullus 234' parental cultivar (2n=20)(Figure 2.4, Figure 2.5 and Figure 2.6). Similar results were reported by Sibi (1976).

To examine for possible DNA sequence variations in the resistant somaclones, RAPD PCR was carried out. In our initial attempts to optimize RAPD PCR analysis in asparagus, only one concentration at 1.5 mM of $MgCl_2$ gave rise to PCR products (data not shown). Reproducible RAPD products were obtained by using T4 gene 32 protein (gp32). In the absence of gp32 in the reaction mixture, the yield of products and the

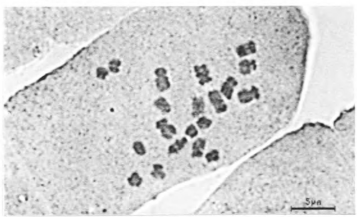

Figure 2.4. The chromosomes in *A. officinalis* cv. Lucullus 234 (2n = 20).

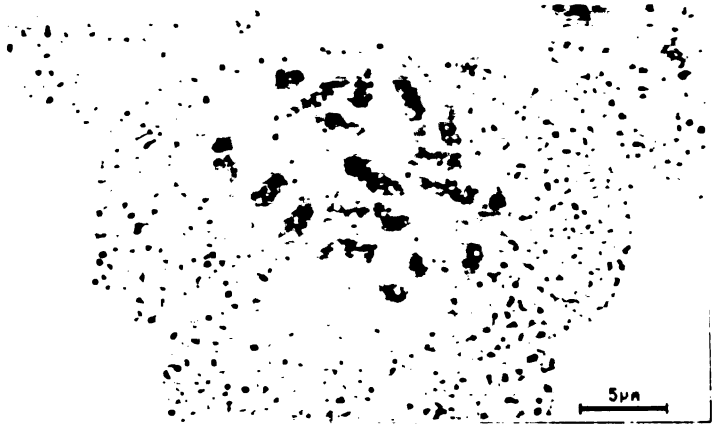

Figure 2.5. The chromosomes in the resistant somaclone R7 of *A. officinalis* cv. Lucullus 234 (2n = 20).

Figure 2.6. The chromosomes in the resistant somaclone R4 of *A. officinalis* cv. Lucullus 234 (2n = 20).

number of bands varied between PCR reactions, especially in the lower molecular weight range (data not shown). An improvement in the yield and reproducibility of PCR products by gp32 has also been reported by Schwarz *et al.* (1990), Panaccio and Lew (1991) and Collins and Symons (1993).

The resistant somaclonal lines R7 and R4 developed from the second cycle of screening and four 'Lucullus 234' seed plants (controls) were screened for RAPDs using 20 arbitrary primers. Of the 20 primers, 75% them generated unique amplification products in R7, that were not found in the four control plants (Figure 2.7, Figure 2.8 and Table 2.7). Thirty five percent of the 20 primers produced polymorphisms in R4 in which the primers E, R and T generated unique amplification products only in R4, but not in the controls, and the primers B, D, F, and G generated unique amplification products only in the controls, but not in R4 (Figure 2.9 and Table 2.7). From one to four unique banding patterns were found in R7 and R4 with each primer (Table 2.7). These results indicate that in the somaclonal lines, R7 and R4, which demonstrated higher levels of *Fusarium* resistance, carried sequence variations unique to the parental source.

The DNA sequence variations found in R7 and R4 may have been recovered from either tissue culture because the culturing techniques induced mutations or variant cells that were already preexisting in somatic tissues. Somaclonal variation could affect *Fusarium* resistance in several possible ways. The resistance could be due to mutations resulting in alternation of gene expression and protein production, for example, changes in base sequence may result in a more resistant enzyme. Alternatively, increase of the resistance could be caused by amplification of a naturally existing resistant gene in

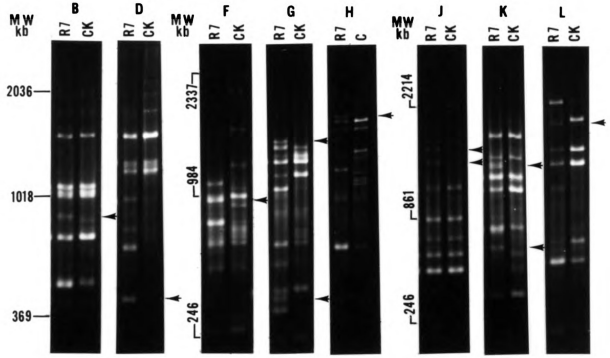

Figure 2.7. Unique polymorphisms found in the resistant somaclone R7 when compared to 'Lucullus 234' parental plants (primers from B to L). Each set of two lanes results from PCR amplification with a single primer. In each set, the first lane contains R7 and the second lane contains one of four 'Lucullus 234' parental plants grown from seed (CK). The arrows represent unique amplification profiles that were consistently present in the resistant lines and absent from the controls.

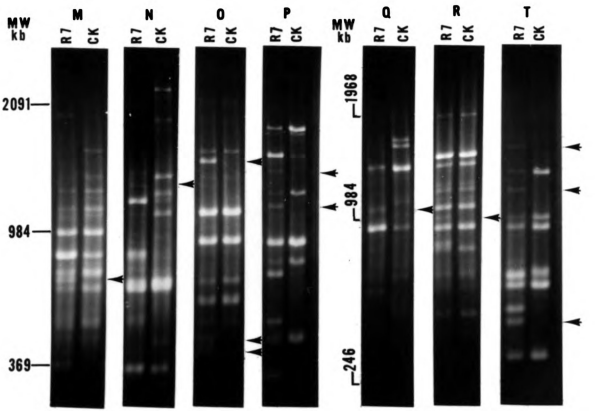

Figure 2.8. Unique polymorphisms found in the resistant somaclone R7 when compared to 'Lucullus 234' parental plants (primers from M to T). Each set of two lanes results from PCR amplification with a single primer. In each set, the first lane contains R7 and the second lane contains one of four 'Lucullus 234' parental plants grown from seed (CK). The arrows represent unique amplification profiles that were consistently present in the resistant lines and absent from th

Table 2.7. Number of unique amplification products that were all present in the resistant somaclone R7, and present (P) or absent (A) in the resistant somaclone R4 when compared to 4 control plants.

Primer	No. of unique amplification products		No. of RAPD Profiles[x]
	Somaclone R7	Somaclone R4	
B	1	1 (A)	3
D	1	1 (A)	3
E	0	1 (P)	4
F	1	1 (A)	3
G	2	1 (A)	3
H	1	0	3
J	2	0	3
K	2	0	4
L	1	0	4
M	1	0	4
N	1	0	4
O	3	0	4
P	2	0	4
Q	1	0	3
R	1	1 (P)	3
T	3	1 (P)	3
Percent of the 20 primers producing polymorphic bands	75%	35%	

[x]RAPD profiles were obtained from two different genomic DNA isolations.

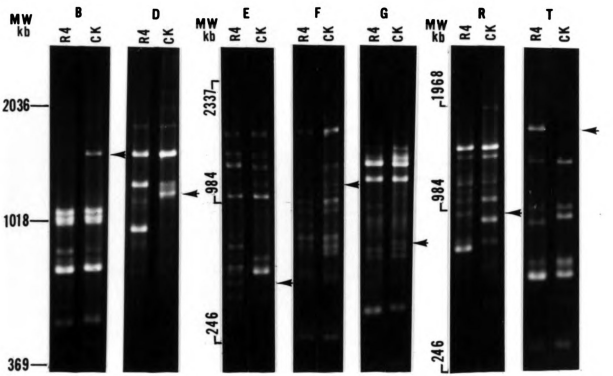

Figure 2.9. Unique polymorphisms found in the resistant somaclone R4 when compared to 'Lucullus 234' parental plants. Each set of two lanes results from PCR amplification with a single primer indicated by a letter. In each set, the first lane contains R4 and the second lane contains one of four 'Lucullus 234' parental plants grown from seed (CK). The arrows represent unique amplification profiles that were present only in the resistant lines or the controls.

'Lucullus 234', although the resistant gene in 'Lucullus 234' has not been determined.

Resistant somaclonal mutant lines, R7 and R4 described here could provide a source of increased resistance to *F. oxysporum* and *F. proliferatum* in an asparagus breeding program. In further experiments, the resistant somaclonal lines, R7 and R4, could be crossed with a susceptible asparagus cultivar and their progenies could be checked for segregation of resistant and susceptible plants to determine if the somaclonal lines have resistant gene. If a resistant gene exists, the RAPD marker described above may be useful to isolate the resistance gene by chromosome walking, or as a genetic marker which can be used in backcross breeding.

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APPENDIX

APPENDIX

The Transformation of Asparagus with A Synthetic Gene Encoding A Novel Lytic Peptide

ABSTRACT

Cut spear fragments from the monocotyledonous plant *Asparagus officinalis* cv. Lucullus 234 were infected with *Agrobacterium tumefaciens* strain LBA4404 harboring an antibacterial and antifungal gene 'Shiva-1', and two reporter genes GUS and NPTII. Inoculated tissues selected on kanamycin-containing medium gave rise to antibiotic resistant calli. Plants were regenerated from the resistant calli and one transgenic plant (T11) was obtained. This transgenic plant (T11) expressed the GUS marker enzyme and Southern blot suggested the presence of two copies of Shiva-1 gene integrated into its genom. Invitro antibacterial assay showed that T11 significantly inhibited the growth of *E. coli* when compared to a control nontransgenic plant and heat treated T11 indicating possible production of Shiva-1 peptide in T11. T11 was then vegetatively micropropagated. The micropropagated plants of T11 had significantly greater resistance to *Fusarium oxysporum* in the greenhouse in comparison with the vegetatively micropropagated nontransgenic plants.

INTRODUCTION

The distinctive feature of the *Agrobacterium* infection process is the natural ability of this pathogen to export oncogenes and other DNA to a wide range of dicotyledonous and monocotyledonous plant species (Binns and Thomashow, 1988; Ream, 1989; Zambryski, 1989; Kado, 1991; Winans, 1992). *Agrobacterium* is a member of the alpha subdivision of the class Proteobacteria and gram negative rods which belong to the family of Rhizobiaceae. It has been divided into five species: *radiobacter*, *tumefaciens*, *rhizogenes*, *rubi*, and *vitis* (Ophel and Kerr, 1990). The *Agrobacterium* which can cause the crown gall on plants has been classified as the species *A. tumefaciens*. When virulent strains of *A. tumefaciens*, harboring a large tumor-inducing (Ti) plasmid, infect wounded plant tissue, a specific segment of the Ti plasmid, T-DNA, transfers into plant cells and stably integrates into plant nuclear DNA. Subsequent expression of the T-DNA genes leads to production of enzymes for biosynthesis of auxin and cytokinin, thereby causing transformed cells to grow as crown gall tumors and to produce opines which are used as carbon and nitrogen source by the bacterium for its growth (Petit *et al.*, 1983). The infection and T-DNA transmission (transfer and integration) processes involve following steps: 1) attraction of *Agrobacterium* cells to the wounded site of plants by chemotactic agents produced by wounded plants, 2) attachment of *Agrobacterium* to the plant cells promoted by polysaccharide controlled by the virulence genes on the chromosome of the bacterium, 3) induction of expression of specific *vir* genes by phenolic compounds produced by the wounded plants,, 4) generation of a T-DNA copy, 5) Formation of a T-strand protein complex, 6) movement of the T-complex through the bacterial membranes, 7) targeting of the T-complex into and within the plant cell, 8) targeting of the T-

complex to the plant cell nucleus, 9) integration of the T-strand into plant cell DNA (Zambryski, 1992). T-DNA transmission involves specific DNA-protein interactions. The right 23-base-pair direct repeats (Shaw *et al.*, 1984; Wang *et al.*, 1984; Peralta and Ream, 1985; Hepburn and White, 1985) as well as a specific flanking sequence of T-DNA (Jen and Chilton, 1986; Peralta *et al.*, 1986; Rubin, 1986; Ji *et al.*, 1988) are required for efficient transmission of T-DNA. The T-DNA transmission also requires virulence genes located outside of the T-DNA region on the plasmid (*vir*) (Garfinkel and Nester, 1980; Klee *et al.*, 1982; Klee *et al.*, 1983; Hille *et al.*, 1984; Stachel and Nester, 1986) and on the *Agrobacterium* chromosome (*chv*, *psc*, *exo*, *att*) (Dylan, *et al.*, 1986; Cangelosi *et al.*, 1987; Marks *et al.*, 1987; Matthysse, 1987; Thomashow *et al.*, 1987; Robertson *et al.*, 1988; Zorreguieta *et al.*, 1988). The mechanism of the last step of T-DNA transmission is not yet clear.

Because of this unique feature of *Agrobacterium*, the *Agrobacterium*-plant DNA transfer system is widely used for plant molecular and genetic engineering experiments. Numerous genes have been successfully introduced into economically important agricultural crops. Of these genes, four types have gained worldwide interest as they improves plant agronomic traits. They are genes conferring: 1). improvement of crop quality, 2). resistance to insects, 3). resistance to herbicide, 4). resistance to plant pathogens (disease resistance) including viruses, fungi, and bacteria.

Transgenic tomato, called Flavr Savr, is engineered by antisense gene of ACC synthase to block ethylene production and delay the onset of fruit ripening. Therefore, this tomato can ripen on the vine and still be firm enough to ship so that they take up more sugar and acids that can improve flavor (Sheeny *et al.*, 1988; Klee *et al.*, 1991;

Van Brunt, 1992).

A synthetic gene encoding the CryIA(b) protein derived from *Bacillus thuringiensis* was introduced into maize. Transgenic maize exhibited excellent resistance to heavy infestation of European corn borer in the field (Koziel *et al.*, 1993).

Tobacco, potato and tomato were transformed with the *bar* gene which confers resistance in *Streptomyces hygroscopicus* to herbicide of bialaphos. Transgenic plants showed complete resistance towards high doses of the commercial formulations of phosphinothricin (PPT) and bialaphos (DeBlock *et al.*, 1987).

Promising progress have been made with field resistance to tobacco mosaic virus in transgenic tomato (Nelson *et al.*, 1988) and to cucumber mosaic virus in transgenic cucumbers (Gonsalves *et al.*, 1992). Transgenic tobacco, canola, and *Brassica napus* with enhanced resistance to fungal pathogen *Rhizoctonia solani* have been developed (Broglie *et al.*, 1991). Transgenic potato expressed an antibacterial gene (Shiva-1) and increased resistance to bacteria (Destefano-Beltran *et al.*, 1990).

Antibacterial proteins, named cecropins, produced by the giant silk moth (*Hyalophra cecropia*) were discovered by a group in Sweden (Boman and Steiner, 1981; Steiner *et al.*, 1981; Andreu *et al.*, 1987). The peptides consist of 35-37 amino acid residues, and comprise three major forms: A, B, and D. A primary mode of action of the proteins is membrane disruption and subsequent lysis due to the bacterial's loss of osmotic integrity (Christensen *et al.*, 1988). Additional research has shown that the peptides enhance overall resistance against fungal diseases and nematodes but normal mammalian and plant cells are not affected (Jaynes *et al.*, 1987, 1989a and 1989b; Destefano-Beltran *et al.*, 1990). Several analogues of cecropins were synthesized by Dr. Jaynes and associates to

test the hypothesis that the biological activity of the peptide is dictated by its physical structure and not by its primary sequence and to obtain proper expression of Shiva-1 in plants. One analogue in particular, Shiva-1, is derived from cecropin B protein which has 36 amino acids. Shiva-1 has 47% amino acid homology with Cecropin B, but has increased biological activity. The size of Shiva-1 gene is 0.125 kb, and it encodes a 38 amino acid long peptide (Destefano-Btran, 1990; Nagpala, 1990).

Cultured stem fragments from *A. officinalis* infected by the oncogenic *A. tumefaciens* strain C58 harboring the wild-type nopaline Ti plasmid developed tumorous proliferations. This tissue was propagated in vitro on hormone-free medium. T-DNA-encoded markers nopaline and agrocinopine were detected in these tissues. However, transgenic plant was not regenerated (Hernalsteens *et al.*, 1984). Later, the same group reported regeneration of transgenic plants of *A. officinalis* after spear segments were infected by *A. tumefaciens* strain C58C1 harboring a nononcogenic Ti plasmid-derived vector containing a chimeric aminoglycoside phosphotransferase [NOS-APH(3') II] gene (kanamycin resistant gene) (Bytebier *et al.*, 1987). Recently, long term embryogenic callus were infected by nononcogenic *A. tumefaciens* strain C58 harboring GUS and NPTII marker genes. One transgenic plant was obtained and confirmed by Southern blot (Delbreil *et al.* (1993).

This appendix reports: 1) the development of the transformation of *A. officinalis* cv. Lucullus 234 using the *A. tumefaciens* strain LBA4404 harboring Shiva-1 gene and regeneration of a transgenic plant (T11), 2) the verification of the transformed plant by the Fluorometric assay, NPTII Elisa and DNA analysis by Southern blot, 3) test of possible production of Shiva-1 peptide in T11 by in-vitro antibacterial-assay against *E.*

coli, 4) the evaluation of the vegetatively micropropagated plants of transgenic plant T11 in response to *F. oxysporum* isolate FOA50 in the greenhouse.

MATERIALS AND METHODS

Plant transformation with *Agrobacterium* Greenhouse-grown spears of *A. officinalis* L. cv. Lucullus 234 were surface-sterilized for 30 min in a 20% aqueous bleach solution (5.25% sodium hypochlorite, Big Chief Bleach, Patterson laboratories, Detroit MI) plus 3mml per liter of Tween-20 (Sigma Chemical Co., St. Louis MO) and cut into 0.5-cm-long pieces. The pieces were precultured for 1 day on tobacco feeder plates containing 1 ml of tobacco suspension culture and covered with a piece of sterile Whatman filter paper on MSF medium (Elmer *et al.*, 1989). The spear segments were incubated by immersing them in an overnight culture at 10^8 cfu/ml of *A. tumefaciens* strain LBA4404 for 30 min. This *Agrobacterium* strain contains a pBI121 plasmid that has a Shiva-1 gene and two reporter genes (NPTII gene for kanamycin resistance and GUS gene) (Figure A.1) (from Dr. Jaynes, Louisiana State University). The explants were then blotted dry, replaced on the tobacco feeder plates for a 2 day co-culture period under dark condition, and transferred to callus-inducing medium (MSF) with 500 mg/l carbenicillin to kill *Agrobacterium* and 100 mg/l kanamycin to select transformed tissue and grown under dark condition for 1 to 2 months. Kanamycin-resistant calli with shoot primordia were transferred to regeneration medium containing a hormone-free Murashige & Skoog (MS) medium (1962) supplemented with 500 mg/l carbenicillin, and plants were regenerated at 27° C with 40 $\mu Em^{-2}s^{-1}$ light and a 16 h photoperiod. The plants were

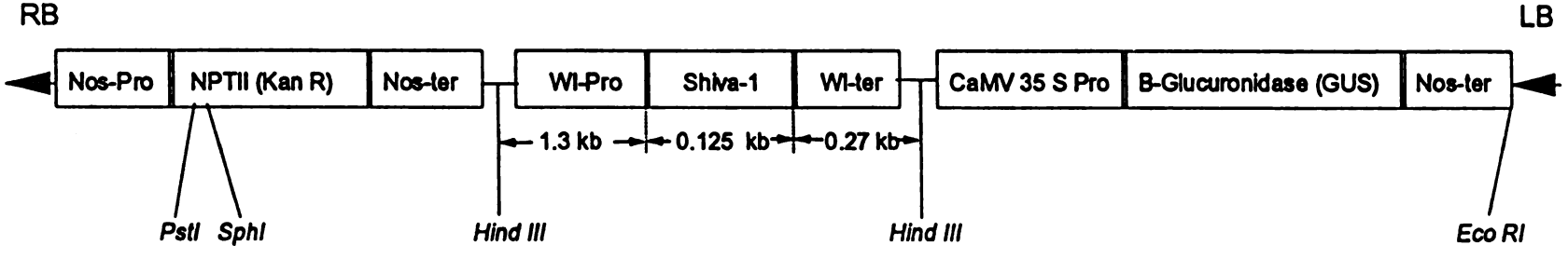

Figure A.1. Restriction map of the T-DNA region of pBI121 plasmid in *A. tumefaciens* LBA4404. Shiva-1 gene was put under the control of the wound-inducible potato proteinase inhibitor II promoter and terminator.

transferred to the greenhouse after acclimazation (Chapter II).

Selection of transformants using Fluorimetric GUS assay and NPTII Elisa

Fluorimetric GUS assay procedure was followed according to Jefferson (1987). Asparagus plant roots were homogenized in GUS extraction buffer (50 mM $NaPO_4$, pH 7.0, 10 mM deta-Mercaptoethanol, 10 mM Na_2EDTA, 0.1% sodium lauryl sarcosine, 0.1% Triton X-100) by grinding in Eppendorf tubes with little disposable pestles. Two hundred and fifty μl assay buffer (GUS extraction buffer with 1 mM 4-methyl umbelliferyl beta-D-glucuronide) was incubated at 37° C to pre-warm and 25 μl of extract was added to 250 μl GUS assay buffer, 100 μl mixture were removed, and each aliquot was added to 0.9 ml stop buffer (0.2 M Na_2CO_3) at 1, 3, and 5 hour, and overnight intervals. The methyl umbelliferone (MU) concentration was determined with Spectro-fluorimeter, excitation at 365 nm, emission at 455 nm.

For the detection and quantitation of neomycin phosphotransferase II (NPT II) protein in crude cellular extracts, NPT II Elisa kit (5 Prime → 3 Prime, Inc., Boulder CO) was used. Asparagus plant shoots were ground in Eppendorf tubes containing 500 μl of cold 0.25 M Tris-Cl, pH 7.8 and 1 mM phenylmethyl-sulfonyfluoride at 4° C. The homogenates were centrifuged at 7500 x g at 4° C for 30 min, and the protein concentrations were determined according to Bradford method (Sambrook *et al.*, 1989). Two hundred μl of diluted Coating Antibody were added to each well of microwell strips, and the microwell strips were covered with parafilm and incubated at 37° C for 2 h. Each well was washed with 1X phosphate buffered saline (PBS) for 3 times, filled with 400 μl of 1X blocking/dilution buffer, incubated at room temperature for 30 min,

and was washed 5 times with 1X washing buffer. Two hundred μl of negative control and transformed plant extract proteins at concentration of 400 μg/ml were added to the each well, and the wells were incubated at room temperature for 2 h. Each well was washed 5 times with 1X washing buffer, filled with 200 μl of biotinylated antibody to NPT II, and incubated 1 h at room temperature. The wells were washed 5 times with 1X washing buffer, and 200 μl of streptavidin conjugated alkaline phosphatase dilution were added to each well. The wells were incubated 30 min at room temperature. Each well was washed 5 times with 1X washing buffer, filled with 200 μl of substrate solution (5 mg p-nitrophenyl phosphate in 2.5 ml of diethanolamine buffer), incubated at room temperature for 30 to 40 min in the dark, and read at 415 nm against the reagent blank in a microtiter well reader. All experiments were designed as completely randomized design and conducted at least two times. Data were subject to ANOVA.

Confirmation of transformed plants by Southern Blot To determine integration of Shiva-1 gene into asparagus plant genome, 5 putative transformed plants (T3, T7, T11, T13, T16), which showed higher GUS activity or higher NPTII protein when compared with control plants, were used for Southern blot. Asparagus shoot DNA was isolated using the procedure of Dellaporta *et al.* (1985). *A. tumefaciens* plasmid DNA (pBI121 plasmid) was isolated and purified from *E. coli* according to method for large-scale preparation of plasmid DNA (Sambrook *et al.*, 1989).

The DNA samples were digested with HindIII at 37°C overnight, and 5 units of HindIII were used for every 1 μg plant genomic DNA. The digested DNA fragments were electrophoretically separated on 1.0% agarose gel at 60 voltage for 5 h. The gel

was denatured in 1.5 M NaCl and 0.5 M NaOH with agitation for 2 X 15 min, and neutralized by soaking in 0.5 M Tris, pH 7.4 and 1.5 M NaCl for 2 X 15 min.

The DNA fragments were transferred to Nytran membrane in 10 X SSPE by the standard capillary blotting. SSPE was prepared according to Sambrook *et al.* (1989). After blotting, the membrane was washed in 5 X SSPE for 5 min at room temperature, dried at room temperature for 30 min and baked under vacuum oven at 80°C for 30 min.

The Shiva-1 probe was made by isolating 1.7 kb Shiva-1 gene fragment from agarose gels with NA-45 DEAE membrane (Schleicher & Schuell, Lowell MA) according to Sambrook *et al.* (1989). The Shiva-1 probe was radioactively labelled with α^{32}P dCTP using DNA labeling kit (5 Prime → 3 Prime, Inc., Boulder CO) following Feinberg and Vogelstein's method (1983). The labelled probes were then purified using Sephadex G-50 column according to Maniatis *et al.* (1982).

The membrane was prehybridized in a buffer (0.25 ml/cm^2) containing 6 X SSPE, 10 X Denhardt's solution, 0.5% SDS, and 100 μg/ml denatured sonicated salmon sperm DNA for a minimum of 2 h at 42°C. The prehybridization solution was completely removed, hybridization solution (0.25 ml/cm^2) was added, and the labelled Shiva-1 probe then was added to the solution and mixed thoroughly. The hybridization was carried out at 65°C for overnight.

The membrane was washed in several hundred milliliters of 2 X SSC and 0.5% SDS at room temperature for 5 min, of 2 X SSC and 0.1% SDS at room temperature for 5 min, of 0.1 X SSC and 0.5% SDS for 30 min at 37°C with gentle agitation, and of 0.1 X SSC, 0.5% SDS for 30 to 60 min at 65°C. The radioactivity in the membrane was monitored after 15 to 30 min using a-hand-held minimonitor to determine how much

longer the membrane need to be washed. The membrane was washed in 0.1 X SSC at room temperature for 2 min and exposed to X-ray film at -70°C with an intensifying screen for one to two weeks.

In-vitro anti-bacterial activity assay of transgenic plant T11 To test possible production of Shiva-1 peptide in T11, an anti-bacterial activity assay of T11 was carried out. The effect of protein extract from T11 and untransformed plants on growth of *E. coli* was tested to determine antibacterial activity directly. *E. coli* strain DH5α was used in the assay because *E. coli* is one of the most Shiva-1 susceptible bacteria (Nagpala, 1990).

For isolation of the total proteins of T11 and control plant, the roots were homogenized in 0.01 M phosphate buffer (pH 6.8) and a little sand with a sterile mortar and pestle on ice. The homogenates were centrifuged for 10 min at 10,000 g, and the supernants were collected. The protein extracts were sterilized by passing through a 0.22 μm filter and the protein concentrations were determined at 0.66 mg/ml according to Bradford method (Sambrook *et al.*, 1989).

For anti-bacterial activity assay, the procedure used was a modification of the method of Destefano-Betran, (1991). *E. coli* bacteria were cultured overnight in 200 ml LB medium at 37°C and 220 rpm, and the O.D. at 600 nm of the culture was determined using a spectrophotometer to control relative uniform bacterial concentrations from one assay to another. Twenty μl of the bacterial culture was added to 1 ml of each of the protein extracts of T11 and control plant, and the heat treated protein extract of T11 by boiling for 5 min. The treatment of boiling protein extract of T11 was to confirm that

the anti-bacterial activity of T11 was possiblely due to the Shiva-1 peptide produced in T11, and not to any possible contaminant. The mixtures were incubated at 37°C and 220 rpm for 1 hour. After the incubation, each mixture was diluted from 10^{-1} to 10^{-5} in 0.01 M phosphate buffer (pH 6.8), and 100 μl of each of the dilutions was cultured on LB agar medium at 37°C for overnight. The colonies were counted, and percentage of survival was determined as the numbers of colonies in each treatment / number of colonies in control. All experiments were conducted as a completely randomized design with two replicate plates per treatment and were conducted at three different times. All the data in the table 3.3 were analyzed by ANOVA and Duncan's Multiple Range Test was used to separate means.

Evaluation of the vegetatively micropropagated plants of T11 in response to *F. oxysporum* in the greenhouse The transgenic plant T11 was vegetatively micropropagated following the method described in chapter II in order to conduct the screening tests with *F. oxysporum*. A cultivar of an ornamental asparagus species *A. densiflorus*, 'Sprengeri', was used as resistant control and a cultivar of *A. officinalis*, 'UC157', as susceptible control for the screening tests. Twelve of one-month-old greenhouse-grown transplants of each of the micropropagated transgenic and nontransgenic plants, and control seedling plants were inoculated with the most virulent isolate of *F. oxysporum* (FOA50) using the method of Wacker *et al.* (1990), and grown in the greenhouse for 1 month. Plants were fertilized once a week (Peters 20N-20P-20K, 200 ppm). The individual plants were rated for the severity of FOA50 infection according the visual rating scale of Stephens *et al.* (1989) and Wacker *et al.* (1990): 1 =

healthy plant, no evidence of disease (highly resistant), 2= few root lesions, <25% diseased (resistant), 3= moderate number of root lesions, <50% (moderately susceptible), 4= many root lesions, <75% (susceptible), and 5= all roots flaccid or dead (highly susceptible). The experiments were designed as complete randomized block design under a natural photoperiod at 25-30°C. Data were subjected to ANOVA and Duncan's Multiple Range Test.

RESULTS AND DISCUSSION

Regeneration and selection of transgenic plants After cut spear segments inoculated with *A. tumefaciens* LBA4404 were cultured on MSF medium, kanamycin-resistant calli with shoot primordia developed within 1 to 2 months. Shoots formed on the kanamycin-resistant calli after 1 to 2 month culture on hormone-free MS medium without kanamycin. Kanamycin inhibited further shoot development in our experiments. Plants were further regenerated from the kanamycin-resistant calli on the hormone-free MS medium, transferred into pots, and grown in the greenhouse.

To screen for transformants, the root tissues of putative transformed and non-transformed (control) plants grown in greenhouse were tested for GUS activity by Fluorimetric assay. Significantly higher GUS enzymatic activity was obtained in three putative transformed plants T3, T11 and T13 when compared with control plant (Table A.1).

NPTII Elisa was also used to select transformants by detection and quantitation of NPTII protein level. NPTII protein level was significantly higher in three putative

Table A.1. Detection and quantitation of GUS activity of putative transgenic plants (PTP) and non-transformed plants by fluorometric assay.

Plants	GUS activity (nM MU/min/g fresh root tissue)[1]
PTP 11	134.0 A
PTP 13	131.7 A
PTP 3	128.4 A
PTP 7	88.0 AB
PTP 2	87.8 AB
PTP 10	87.6 AB
PTP 12	77.3 B
PTP 9	72.4 B
PTP 4	71.5 B
PTP 6	68.0 B
PTP 1	62.5 B
Non-transgenic plant	50.5 B

[1]Means within a column with different letters are significantly different (Duncan's multiple range test, $P<0.05$).

transformed plants T1, T7, and T13 than that in non-transformed plants (Table A.2). One putative transformed plant T13 expressed both GUS and NPTII marker enzymes. The putative transformed plant T3 and T11 had significantly higher GUS activity, but not NPTII protein. One explanation for this could be that NPTII gene in these two plants expressed at low level, and NPTII Elisa is not sensitive enough to detect it. Alternatively, the NPTII gene was not inserted into the genomes of the plants due to internal deletion of the integrated T-DNA. Internal deletion of the integrated T-DNA in transgenic petunia has been reported by Deroles and Gardner (1988). Another alternative explanation could be that NPTII gene was inactivated by methylation. Methylation of wild type-T-DNA occurred and methylation of regulatory sequences may partially or fully inactivate gene expression. DNA methylation in plants has been reported and shown to be inversely correlated to gene expression (Amasino *et al.*, 1984; Van Slogteren *et al.*, 1984). The same explanation could apply to putative transformed plant T1 and T7 which showed significantly higher NPTII protein, but not GUS activity.

Confirmation of transgenic plants To confirm that Shiva-1 gene integrated into genomes of transgenic plants, five pre-selected putative transformed plants T3, T7, T11, T13 and T16 by Fluorimetric assay and NPTII Elisa were analyzed by Southern blot. Based on the restriction map of pBI121 plasmid (Figure A.1), it can be predicted that this plasmid give only one band 1.7 kb in length when digested by HindIII and using the 1.7 kb Shiva-1 DNA as a probe. Two bands with the expected 1.7 kb HindIII Shiva-1 containing fragment were obtained in T11 (Figure A.2). No hybridization by 1.7 kb Shiva-1 probe was observed with DNA extracted from either untransformed plant or

Table A.2. Detection and quantitation of NPTII protein in crude cellular extracts of putative transgenic plants (PTP) and non-transgenic plants by NPTII Elisa.

Plants	NPTII protein(pg)[1]
PTP 13	18.4 A
PTP 1	10.1 B
PTP 7	7.0 BC
PTP 16	5.6 CD
PTP 21	5.3 CDE
PTP 23	3.5 DEF
PTP 18	3.2 DEF
PTP 19	2.9 DEF
PTP 15	2.5 DEF
Non-transgenic plant	2.4 DEF
PTP 9	1.7 EF
Non-transgenic plant	1.5 F
PTP 14	1.5 F
PTP 10	1.2 F
PTP 22	1.2 F
PTP 20	1.0 F
PTP 2	0 F
PTP 17	0 F
PTP 6	0 F
Non-transgenic plant	0 F
PTP 3	0 F
PTP 4	0 F
PTP 11	0 F
Non-transgenic plant	0 F

[1]Means within a column with different letters are significantly different (Duncan's multiple range test, $P<0.05$).

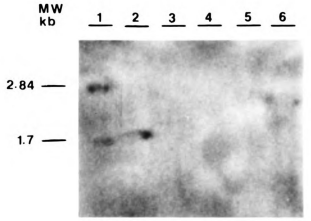

Figure A.2. Autoradiogram of Southern blot of DNA extracted from transformed and untransformed plants, digested with restriction enzyme HindIII and membrane hybridized with 1.7 kb Shiva-1 probe. Lane 1 to 4: transformed plants T11, T7, T13 and T16. Lane 6: untransformed plant (control).

other putative transformed plants (Figure A.2). The occurrence of two bands in T11 may be caused by two integrations of Shiva-1 gene in two loci in one chromosome or in different chromosomes with rearrangement of the T-DNA and/ or methylation of the T-DNA sequences especially those in HindIII sites. Truncation, rearrangement, repetition or methylation of the introduced T-DNA have been reported in transgenic petunias (Jones *et al.*, 1987; Deroles and Gardner, 1988) and tobacco (Hobbs *et al.*, 1990). One putative transformed plant T13 had significantly higher GUS activity and NPTII protein level with comparison to untransformed plants, but no hybridization with Shiva-1 probe. No presence of Shiva-1 gene in T3, T7, T13 and T16 may be due to internal deletion of the integrated T-DNA. Deroles and Gardner (1988) reported internal deletion of the integrated T-DNA in transgenic petunia.

Among 16 southern blots of transgenic plants, 2 blots showed expected 1.7 kb HindIII Shiva-1 DNA fragment band in transgenic plant T11. No hybridization by 1.7 kb Shiva-a probe was observed with pBI121 plasmid DNA (positive control) which contains Shiva-1 gene in one blot (data not shown in Figure A.2). This may be explained that the concentration of the plasmid DNA was too low to be detected by this method.

Antibacterial activity of transgenic plant T11 The protein extract from T11 significantly prohibited bacterial growth when compared with untransformed plant extract (Table A.3). Sixty five percent of bacteria survived after they were treated with the protein extract from T11 (Table A.3). The protein extract at concentration of 0.66 mg/ml from T11 had the antibacterial activity equivalent to 0.6 μM pure Shiva-1 peptide

Table A.3. Inhibition of *E. coli* growth by crude protein extracts from transgenic plant T11.

Treatment	Average number of surviving bacteria[1]	Percent of surviving bacteria
Protein extract of transgenic plant 11 (T11) (0.66 mg/ml)	29 A	65.9 A
Protein extract of non-transgenic plant (CK) (0.66 mg/ml)	44 B	100 B
Boiling protein extract of transgenic plant T11 (0.66 mg/ml)	41 B	93.2 B

[1]Means of 2 replicate plates from three replicate experiments, means within a column with different letters are significantly different (Duncan's multiple range test, $P<0.05$).

(Nagpala, 1990). The inhibitory effect of the protein extract from T11 on the bacterial growth was almost completely overcome by boiling the protein extract of T11 (Table A.3). These data indicate that Shiva-1 peptide may be produced in T11.

Evaluation of the vegetatively micropropagated plants of T11 in response to *F. oxysporum* in the greenhouse After vegetatively micropropagated plants of each of T11 and nontransgenic plant were screened with FOA50, the transgenic plants were found to be significantly more resistant to FOA50 than the nontransgenic plants (Table A.4). Of 12 transgenic plants, 58.3% of them were rated at a 1 or 2, whereas 83.3% of nontransgenic plants were rated at a 3 or 4 (Table A.4).

Southern blot suggested that the transgenic plant T11 had two copies of Shiva-1 gene integrated into its genome. In vitro antibacterial activity assay indicated possible production of Shiva-1 peptide in T11. The transgenic plants from vegetative micropropagation showed a significant increase in resistance to FOA50 in the greenhouse. The Shiva-1 gene is under the control of the wound-inducible potato proteinase inhibitor II promoter. The proteinase inhibitor II promoter is usually turned on by severe wounding through mechanical means or insect injury (Thornburg *et al.*, 1987). *F. oxysporum* enters plants by directly penetrating meristimatic zones of roots using appressoria, by partially digesting root tissue using its enzyme, or through crushed cortical cells by emerging lateral roots (Nelson *et al.*, 1981). It is possible that this promoter was activated by fungal invasion through fungus induced wounding. Transgenic potato plants with Shiva-1 gene under the control of the same promoter showed that the expression of Shiva-1 gene is triggered by mechanical and pathogen

Table A.4. Disease rating of vegetatively micropropagated plants of transgenic plant T11 and nontransgenic plants, and control plants screened with *Fusarium oxyspor um* (isolate FOA50) in the greenhouse.

Plant source	No. of plants in disease rating scales[x]					Mean disease rating[y]
	1	2	3	4	5	
'UC157' (Susceptible control)	0	2	8	2	0	3.0 A
Micropropagated seed plants of 'Lucullus 234'	1	1	7	3	0	3.0 A
Micropropagated plants of transgenic plant T11	4	3	4	1	0	2.2 B
'Sprengeri' (Resistant control)	11	1	0	0	0	1.1 C

[x]Twelve plants of each of plant sources were screened with FOA50 using complete randomized block design. Disease was assessed using a visual rating scale: 1= no disease, 2= < 25% diseased, 3= < 50% diseased, 4= <75% diseased, 5= >75% diseased.

[y]Means of disease rating within a column with different letters are significantly different (Duncan's multiple range test, $P < 0.05$).

induced wounding (Destefano-Beltran *et al.*, 1990).

Asparagus transgenic plants from vegetative micropropagation with enhanced resistance to *F. oxysporum* may be useful for asparagus breeding. Clearly, futher analysis such as Nothern blot and Western blot are necessary to study expression of Shiva-1 gene in T11 and to determine mechanism of the resistance.

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