Arteca R

97-#32

STABY

The Fred C. Gloeckner Foundation Inc.

600 Mamaroneck Avenue • Harrison, NY 10528-1631
Phone (914) 698-2300

Please review the Guidelines on the reverse side of this form before completing the application.

APPLICATION FOR RESEARCH GRANT FROM:

1. TITLE OF PROJECT Genetically Engineering Floricultural Crops to Block Ethylene Perception

2. PRINCIPAL INVESTIGATOR Dr. Richard N. Arteca

3. NAME OF INSTITUTION The Pennsylvania State University

4. MAILING ADDRESS Department of Horticulture

   CITY University Park STATE PA ZIP CODE 16802-4200

   TELEPHONE (814) 863-2252 FAX (814) 863-6139

5. LOCATION OF STUDY 211 Tyson Building

6. TOTAL AMOUNT REQUESTED FOR 12 MONTH PERIOD (usually September 1 to August 31)

   $ 9,500 **(Include a general itemized budget).**

7. ANTICIPATED DURATION OF THE PROJECT 3 years

8. OTHER SOURCES (Foundations, Etc.) AND AMOUNTS FOR WHICH A SIMILAR PROPOSAL HAS BEEN SUBMITTED None

9. PURPOSE FOR WHICH FUNDS ARE REQUESTED (Use separate pages if additional space is required).

   To partially support a graduate student and for materials and supplies

10. MAKE A BROAD STATEMENT OF THE FOLLOWING ITEMS
    A. Research objectives and justification
    B. Review of key literature
    C. General outline of materials and methods
    D. Anticipated benefit to the floriculture industry

(1) Quest Floriculture — Poor plants
(2) Good Lab work
(1) Good Idea

11. PROJECT LEADER QUALIFICATIONS In a paragraph or two, state the training/expertise that qualifies you for a grant to work in this research area. Extensive vitae information and long autobiographies are not necessary.

Genetically Engineering Floricultural Crops to Block Ethylene Perception

Submitted by:
The Pennsylvania State University
College of Agricultural Sciences
Department of Horticulture

Submitted to:
The Fred C. Gloeckner Foundation
600 Mamaroneck Avenue
Harrison, NY 10528-1631

Submitted by:

Richard N. Arteca
Professor of Hort. Physiology
Department of Horticulture
The Pennsylvania State University
University Park, PA 16802
814/863-2252 (Telephone)
814/863-6139 (FAX)

Approved by:

E. J. Holcomb
Professor and Head of Horticulture

Research Objectives and Justification

Ethylene has been shown to have a major role in decreasing longevity in floricultural crops. It is an air pollutant commonly encountered by plants from the time they are put into shipping channels through their final destination which is the home environment. Physical and environmental stresses to the plant cause an increase in production of "stress ethylene". In recent years two important regulatory genes (ACC synthase and ACC oxidase) encoding for enzymes in the ethylene biosynthetic pathway have been identified in a variety of crops. The identification of these genes has enabled researchers to regulate ethylene production by reducing stress-induced ACC synthase or ACC oxidase at the molecular level by reducing their expression using antisense technology. However, simply blocking ethylene biosynthesis in the plant does not protect it from ethylene encountered in the environment. In this study we are proposing to identify the gene encoding the ethylene receptor (*ETR*) in *Pelargonium x domesticum,* this will enable us to create plants which are insensitive to ethylene. We will accomplish this by incorporating the antisense *ETR* gene into *Pelargonium x domesticum* using a novel transformation and regeneration system developed in our laboratory. Upon completion of this study transgenic plants will be produced with reduced ethylene sensitivity, thereby, reducing injury caused by ethylene which typically occurs between the wholesaler and the home environment. The information gained from this study in *Pelargonium* has the potential to be applied to other floricultural crops.

Review of Key Literature

The tendency of physical and environmental stresses to accentuate the production of ethylene by plant tissues has given rise to the term "stress ethylene". During transportation from the wholesale grower to the retailer, retailer display and storage, followed by final transportation to the consumer's home environment, many types of stress occur which can stimulate ethylene production. In addition to plant derived ethylene production, it is known that ethylene is a common pollutant in our atmosphere commonly produced by fires, automobiles, industry and others (Abeles et al., 1992). Although the wholesale grower produces a high quality product, little control can be exercised over different forms of stress which cuttings or plants encounter nor the level of pollutant which they are exposed to during transportation and at their final destination, the consumers home.

There are several reports in the literature which provide direct evidence that ethylene is the cause of petal shattering and leaf yellowing in *Pelargonium* (Deneke, 1988; Deneke et al., 1990; Evensen, 1991; Armitage et al., 1980; Arteca et al., 1996). It has been reported that both intact and detached inflorescences abscise in response to treatments with 0.5 µl/L of exogenous ethylene for 1 hour (Deneke, 1988; Deneke et al., 1990). Research in our laboratory has shown results similar to Deneke (1988) with a 0.5 µl/L treatment. In addition we were able to demonstrate that when plants were treated at concentrations as low as 0.1 µl/L for 1 hour there was a significant

increase in petal abscission above the control. Ethylene sensitivity has been reported to vary among cultivars with some being more sensitive than others (Deneke, 1988). When florets age there is an increasing sensitivity to ethylene (Deneke, 1988). Silver thiosulfate has been shown to reduce ethylene sensitivity and extend floret longevity beyond that of the controls, however, a number of side effects were observed (Deneke, 1988). A climacteric rise in endogenous levels of ethylene has been shown to occur in *Pelargonium* gynoecia (including the receptacle) as floret age increased. All *Pelargonium* cultivars tested which produce more ethylene have been shown to abscise their petals earlier (Deneke, 1988; Deneke et al., 1990). When unrooted *Pelargonium* cuttings are stored for a five day period there is a climacteric rise in ethylene production. Changes in gene expression of the ethylene biosynthetic enzymes, ACC synthase (*GAC-1* and *GAC-2*) and ACC oxidase (*GEFE-1*) were observed in cuttings stored at 4°C and 25°C and these changes correlated with increases in ethylene and ACC production (Arteca et al., 1996). Based on reports in the literature and findings in our laboratory it appears likely that ethylene regulates petal abscission and a decline in cutting quality since inflorescences are very sensitive to ethylene and increased endogenous ethylene production precedes petal abscission and leaf yellowing.

Although there is a considerable amount of evidence that ethylene is involved in the decline of quality in cuttings and plants during shipment, there are presently no good methods which can be used to overcome its negative effect without adverse side effects. The available approaches for decreasing the adverse effects of ethylene on flower and leaf senescence are reduced temperatures, hypobaric storage, ethylene scrubbers, ethylene action and biosynthesis inhibitors. Storage at 4°C is an unreliable practice for a number of reasons. It has been reported that there is a burst of ethylene after plant tissues are removed from 4°C and placed at higher temperatures. In addition, maintenance of reduced temperatures is not possible once the cuttings or plants leave the wholesale grower and enter the retail outlet and finally to the consumer environment. Hypobaric storage is expensive and not practical for transportation. Ethylene scrubbers have been shown to be ineffective in *Pelargonium* because they do not reduce ethylene contained within the tissues to levels which reduce the senescence process. The use of ethylene action or biosynthesis inhibitors are also ineffective because they are not specific for ethylene and cause numerous adverse side effects; in fact, for this reason they are not commercially used in any crop.

The genes for ACC synthase and ACC oxidase have recently been cloned in several laboratories including ours. We have recently identified two *Pelargonium* ACC synthase cDNAs (*GAC-1* and *GAC-2*) (Wang and Arteca, 1995) and one ACC oxidase (*GEFE-1*) (Wang et al., 1994). At the present time we are attempting to regulate ethylene production in *Pelargonium* by reducing stress induced ACC synthase or ACC oxidase at the molecular level by reducing expression of there respective genes using antisense technology. The use of antisense technology has been successful in delaying fruit ripening in tomato which is very sensitive to ethylene (Oeller

et al., 1991; Hamilton et al., 1990). Climacteric fruit can be induced to ripen by treatment with ethylene at concentrations above 0.1 μL of ethylene per liter of air. It has been shown that the use of antisense technology with the ACC synthase gene can reduce ethylene levels to below 0.1 nL ethylene per gram of fruit mass per hour (Oeller et al., 1991). We have shown that *Pelargoniums* are similar in sensitivity to tomato with 0.1 μl/L ethylene causing petal shattering and leaf yellowing. Therefore, if we are successful in obtaining a similar reduction in ethylene levels in our antisense plants as obtained by Oeller et al. (1991), the deleterious effects of ethylene on *Pelargonium* could possibly be dramatically reduced. However, we may not be successful in our endeavor because once a plant or cutting is placed into shipping channels there is no control over ethylene which is commonly found as an air pollutant. Therefore, in this proposal our approach will be to block ethylene perception. In recent years our understanding of ethylene signal transduction has increased dramatically using a genetic approach with *Arabidopsis thaliana.* A number of mutants affecting ethylene responses have been identified (Ecker, 1995). Using these mutants an *ETR1* gene was identified and evidence has been presented that it encodes for an ethylene receptor. Functional isoforms of *ETR1* have recently been identified in *Arabidopsis* (*ERS*) (Hua et al., 1995) and tomato (*eTAE1*) (Zhou et al., 1996) using *ETR1* as a heterologous probe for library screening. Therefore in the proposed study our strategy will be to identify the homologue *ETR1* in *Pelargonium* using a heterologous probe. If we are unsuccessful using the heterologous *ETR1* cDNA we will clone the gene in *Pelargonium* using sequence information in the literature to produce degenerate primers for PCR production of an *ETR1* homologue in *Pelargonium.* Ethylene is a plant hormone which is involved in normal plant growth and development. Although the question might be raised, if you block ethylene perception by the plant will it die. While this is a good question, mutant plants (not *Pelargonium*) have been generated which are completely insensitive to ethylene and these plants grown normally (Bleecker and Schaller, 1996). Therefore, using the *ETR1* gene to block ethylene perception should not be a problem. Once the proposed research is completed we will be positioned to genetically engineer other floricultural crops to block ethylene perception, thereby, reducing problems with injuries which occur between the wholesaler and the consumer's home where its longevity can be increased.

Materials and Methods

A. Probes for ETR

An *Arabidopsis ETR* cDNA will be used to probe our λgt11 *Pelargonium* cDNA library (Wang and Arteca, 1995). The *Arabidopsis* cDNA has been successfully used to obtain a tomato *ETR* homologue (Zhou et al. 1996). However, if problems arise with the heterologous probe our alternative approach will be to use PCR to produce a *Pelargonium ETR* homologue. RNA will be extracted from the leaves of *Pelargonium* plants subjected to simulated shipping/storage conditions

for 3 days. This RNA will be used to produce a *Pelargonium ETR* using RNA based PCR reactions (Botella et al., 1992). A *Pelargonium* cDNA fragment for *ETR* will be produced using degenerate oligonucleotides encoding TQEETG and VMNHEM as the upstream and downstream primers, respectively. The resulting fragment encoding approximately 30% of the coding region of *ETR* will then be used to screen our λgt11 *Pelargonium* library to obtain a full length cDNA clone.

B. Screening of the λgt11 Pelargonium cDNA library

A cDNA library from the leaves of *Pelargonium* plants subjected to simulated shipping/storage stress (Wang and Arteca, 1995) will be screened using the probes obtained in section A by standard protocols (Sambrook et al., 1989). Filters will be prehybridized at 42°C in prehybridization solution (6x SSC, 5x Denhardt's reagent, 0.1% SDS, 100 μg/ml denatured fragmented salmon sperm DNA, 50% formamide) for 4 hours. Hybridizations will be performed overnight at 42°C using labeled probe. Hybridized filters will be washed at room temperature in 2x SSC, 0.1% SDS for 30 min, then 2 times in 0.2x SSC, 0.1% SDS at 65°C for 30 min each. The dried filters will then be exposed to X-ray film with one intensifying screen overnight at -80°C. Individual positive plaques will be purified by several rounds of plating and hybridization (Sambrook et al., 1989). The insert DNA from positive plaques will be excised and subcloned into pBluescript (SK^-) (Stratagene, La Jolla, CA). Transformants will be selected on LB plates containing ampicillin (50 μg/ml) and X-gal (0.033% w/v). Plasmid DNA will be isolated using the alkaline lysis method.

C. DNA sequence analysis

DNA will be sequenced by an automated ABI 377 Prism sequencer with fluorescent terminators using synthetic oligonucleotides. DNA sequence analysis will be facilitated by the use of the Lasergene package (DNAStar, Madison, WI). The *Pelargonium ETR* gene structure will be compared to existing sequences in the Genbank.

D. Chimeric gene constructs and vectors

The coding region of the *Pelargonium ETR* cDNA will be ligated in the reverse or antisense orientation to the *CaMV* promoter and flanked by the NOS 3' untranslated region and polyadenylation signal, then ligated into pBI101. This vector will be mobilized into *Agrobacterium tumefaciens* strain LB4404 by the triparental conjugation system using the helper plasmid pRK2013. Transconjugants will be selected on LB plates containing kanamycin and streptomycin.

E. Transformation and Regeneration

Petiole sections will be washed in 70% ethanol for 1 minute followed by a 6 minute wash with 10% bleach. They will then be washed 3 times with sterile distilled water and cut into 1-2 cm segments. An *Agrobacterium* culture containing the antisense construct will be added to a petri dish with the cut segments and gently shaken for 5-10 minutes. The segments will then be blotted to remove excess media and placed in a "shooting" media containing kanamycin and carbenicillin to

select for transformed cells. The "shooting" media contains MS salts, vitamins, sucrose, an auxin, a cytokinin and Gelrite as the solidifying agent. Segments will be maintained in the dark at 24°C and transferred to fresh media as needed. The combination of "shooting" media and altering light levels results in the regeneration of plantlets within 1-2 months after infection, with plantlets ranging in size from 0.5 to 2.0 cm tall. Since *Pelargoniums* readily root there will be no need for a rooting media. These plants will be transferred to magenta vessels with vented closures containing a basic media of MS salts, vitamins and sucrose. Within 1-2 months plantlets will then be transferred to soil and gradually acclimated to the greenhouse environment. The regeneration system has been used very successfully in our laboratory and a patent is currently pending jointly with Oglevee Ltd. on this process. Using this system, plantlets can be moved into the greenhouse will minor losses.

F. Confirmation of gene insertion into Pelargonium

The structure and copy number of the Ti plasmid will be confirmed using Southern blot analysis. Genomic DNA will be extracted from *Pelargonium* tissue using the procedure outlined by Wang and Arteca (1995). The DNA (5 μg) will be digested with the appropriate enzymes and fractionated on a 0.6% agarose gel. DNA will then be transferred to a nylon membrane by capillary transfer using 10x SSC overnight. The blot will be prehybridized and hybridized as previously described in section B. The washing steps will be under high stringency conditions. Neomycin phosphotransferase (nptII) assays on transformed plants will be according to the method of McDonnell et al. (1987).

G. Evaluation of transformants

At least 10-15 transformants of each construct will serve as mother plants for cuttings. Unrooted transformed *Pelargonium* cuttings will be removed and subjected to the worst case scenario simulated shipping/storage conditions for 3 days to induce maximal increases in ethylene production as determined by gas chromatography (Arteca et al., 1983). A portion of the tissue will immediately be used for assays described below and another portion rinsed with sterile water then immediately frozen with liquid nitrogen and stored at -80°C until extraction of nucleic acids. Chlorophyll will be extracted in 80% (v/v) acetone and measured by the method of Arnon (1949). In order to evaluate the effects of ethylene petal shattering plants will be grown to flowering using standard protocols and the percentage shattering in response to 0.5 μL/L ethylene will be evaluated.

Anticipated Benefit to the Floriculture Industry

During transportation from the wholesale grower to the retailer, retailer display and storage, followed by final transportation to the consumer's home environment, many types of stress occur which can stimulate ethylene production. In addition to plant derived ethylene production, it is known that ethylene is a common pollutant in our atmosphere commonly produced by fires,

automobiles, industry and others (Abeles et al., 1992). Although the wholesale grower produces a high quality product, little control can be exercised over different forms of stress which cuttings or plants encounter nor the level of pollutant which they are exposed to during transportation and at their final destination, the consumers home. Ethylene has been shown to have a major role in decreasing longevity in floricultural crops. It is an air pollutant commonly encountered by plants from the time they are put into shipping channels through their final destination which is the home environment. Physical and environmental stresses to the plant cause an increase in production of "stress ethylene". In recent years two important regulatory genes (ACC synthase and ACC oxidase) encoding for enzymes in the ethylene biosynthetic pathway have been identified in a variety of crops. The identification of these genes has enabled researchers to regulate ethylene production by reducing stress-induced ACC synthase or ACC oxidase at the molecular level by reducing their expression using antisense technology. However, simply blocking ethylene biosynthesis in the plant does not protect it from ethylene encountered in the environment. In this study we are proposing to identify the gene encoding the ethylene receptor (*ETR*) in *Pelargonium x domesticum,* this will enable us to create plants which are insensitive to ethylene. Upon completion of this study transgenic plants will be produced with reduced ethylene sensitivity, thereby, reducing injury caused by ethylene which typically occurs between the wholesaler and the home environment. The information gained from this study in *Pelargonium* has the potential to be applied to other floricultural crops which will bennefit the industry by decreasing problems associated with ethylene.

Timetable

Objective	**Year** 0	1	2	3
1. Screen λgt11 *Pelargonium* library, identify and characterize *Pelargonium ETR* gene	******			
2. Produce antisense constructs, transform and regenerate *Pelargonium,* verify transformants		******		
3. Evaluate sensitivity to ethylene in transformants			******	

Literature cited

Abeles FB, PW Morgan, ME Saltveit, Jr. 1992. Ethylene in plant biology. Second Edition Academic Press, New York.

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Estimated Budget Submitted by The Pennsylvania State University
to the Fred C. Gloeckner Foundation
September 1, 1997 - August 31, 1998

A. Salaries and Wages	
Prin. Inv. R. N. Arteca	0
Total Category I	0
Wages, technician, $10/hr	5,998
Total Category III	5,998
Total Salaries and Wages	**5,998**
B. Fringe Benefits	
Category I @ 26.65%	0
Category III @ 8.37%	502
Total Fringe Benefits	**502**
C. Total Salaries, Wages, and Fringe Benefits	**6,500**
D. Equipment	**0**
E. Materials & Supplies	**3,000**
F. Travel	**0**
G. Publication Costs	**0**
H. Computer Costs	**0**
I. Other Direct Costs	**0**
J. Total Direct Costs	**9,500**

Budget Notes: JKM:3/10/97

Fringe Benefits: Rates are negotiated and approved by the Office of Naval Research, Penn State's cognizant federal agency. Fixed rates for the period of July 1, 1996 through June 30, 1997 are 26.65% applicable to Category I salaries, 11.71% applicable to Category II graduate assistant salaries, and 8.37% applicable to Category III wages.

BIOGRAPHICAL SKETCH:

Richard N. Arteca
Born: August 23, 1950

ACADEMIC TRAINING:

Utah State University, B.S. in Horticulture, 1972 (minor in Botany)
Utah State University, M.S. in Plant Science, 1976
Washington State University, Ph.D. in Horticulture, 1979

PROFESSIONAL SOCIETIES:

American Society of Plant Physiologists
American Society for Horticultural Science
International Society for Plant Molecular Biology
Plant Growth Regulator Society of America
Gamma Sigma Delta

PROFESSIONAL EXPERIENCE:

July 92 - Present	Professor, Department of Horticulture, The Pennsylvania State University
July 86 - June 92	Associate Professor, Department of Horticulture, The Pennsylvania State University
June 86 - Dec.89	Coordinator of the Centralized Hybridoma Facility, The Pennsylvania State University
Dec. 79 - July 86	Assistant Professor, Department of Horticulture, The Pennsylvania State University

I. Publications (last 5 years)

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PENNSTATE

Department of Horticulture
College of Agricultural Sciences

Phone: 814-865-2571
FAX: 814-863-6139

The Pennsylvania State University
103 Tyson Building
University Park, PA 16802-4200

March 30, 1997

The Fred C. Gloeckner Foundation
600 Mamaroneck Avenue
Harrison, NY 10528-1631

Dear Sirs:

Enclosed please find one original and 9 copies of the project entitled "Genetic Engineering Floricultural Crops to Block Ethylene Perception", by Richard N. Arteca. I would like to take this opportunity to thank the Gloeckner Foundation in advance for its continued support of this new and exciting area of research. If there are any further question please feel free to contact me at 814-863-2252 (telephone) or Rich_Arteca@agcs.cas.psu.edu.

Sincerely,

Richard N. Arteca
Professor of Horticultural Physiology