SUMMARY RESEARCH PROGRESS REPORT FOR 1992
AND RESEARCH PROPOSAL FOR 1993

Submitted to:
SLV Research Center Committee
and the
Colorado Potato Administrative Committe (Area II)

TITLE: Engineering Resistance to Fungal Pathogens in Potato

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PROJECT JUSTIFICATION: The potato (*Solanum tuberosum* L.) is the most important non-cereal food crop in the world. It is the fourth major food crop of the world and is next only to wheat, rice and maize in terms of total food production (Vayda and Park, 1990; Salunkhe and Kadam, 1991). Potato can produce more energy and protein per unit land than any other single food crop (Bajaj, 1987). Because of its immense commercial value, the potato is widely used in research to increase the yield and quality. The potato is susceptible to a great number of diseases, some of which are worldwide importance whereas others are of more localized significance (Rich, 1983). Approximately 160 diseases and disorders of *Solanum tuberosum* have been described (Rich, 1983) and majority of these diseases (about 50) caused are by fungal pathogens. In potato, crop loss resulting from fungal diseases is enormous (Rich, 1983). The most important fungal diseases are caused by *Alternaria solani* (early blight), *Phytophthora infestans* (late blight), *Fusarium spp* (tuber rot), *Rhizoctonia solani* (black scurf), *Verticillium dahliae* (verticillium wilt). A variety of fungicides have been developed and used to control phytopathogenic fungi of potato. The use of these fungicides is moderately successful. However, the use of fungicides is limited to minimum for various reasons. Moreover, variants of fungus that are resistant to fungicides have been found recently (Anderson, 1993).

Recent developments in recombinant DNA technology offer new approaches to develop plants that are more resistant of fungal diseases (see Fraley, 1992, for a review, Broglie, *et al.* 1991; Stark *et al.*, 1992). Many different proteins with antifungal activity have been detected in plants (Roberts, and Selitrennikoff, 1986; Roberts *et al.*, 1988). Recently a new group of proteins called thaumatin-like proteins (permatins) have been shown to have strong antifungal activity (Roberts and Selitrennikoff, 1988; Vigers *et al.*, 1991, 1992; Huynh *et al.*, 1992). These antifungal proteins have been shown to inhibit growth of wide range of agronomically important fungal phytopathogens including *Phytopthera infestans* (Woloshuk *et al.*, 1992), *Alternaria solani* (Huynh *et al.*, 1992), *Fusarium oxysporum* (Huynh *et al.*, 1992). Thaumatin-like proteins appear to be present in all plants and have been characterized from various plant species from both monocots and dicots including potato (King *et al.*, 1986; Singh *et al.*, 1987; Richardson *et al.*, 1987; Pierpoint *et al.*, 1987; Kauffmann *et al.*, 1990; Roberts and Selitrennikoff, 1990; Vigers *et al.*, 1991; 1992; Pierpoint *et al.*, 1990; Rebmann *et al.*, 1991).

The incorporation and overexpression of genes encoding potent anti-fungal proteins in plants may significantly augment the level of their resistance to fungal diseases. Using recently developed methods, genes of choice can be routinely introduced into the genome of

potato plants. In recent years tremendous progress has been made in obtaining plants that are resistant to viruses, herbicides and insects (Cornelissen *et al.*, 1986; Brogile, 1992; Fraley, 1992; Moffat, 1992) by genetic engineering. The aim of this proposal to first isolate complementary DNAs (cDNAs) to thaumatin-like genes from potato and use these cDNAs to obtain transgenic plants that are overproducing these antifungal proteins.

Potato is a good model system both for basic and applied research because: i) it is easy to introduce genes into potato using *Agrobacterium* and generate a large number of transgenic plants (Sheerman and Bevan, 1988; Wenzler *et al.*, 1989a; Mitten *et al.*, 1990), ii) it has relatively small genome (Bennett and Smith, 1976, 1982; Vyada and Park, 1990), and iii) it is one of the major food crops of the world (Vayda and Park, 1990; Salunkhe *et al.*, 1991). Finally, substantial progress in improving potatoes by the use of modern techniques has been realized. Transgenic potato plants, including plants derived from commercial varieties such as Russet Burbank that are resistant to potato virus X and potato virus Y, have been produced (Hemenway *et al.*, 1988; Hoekema *et al.*, 1989; Lawson *et al.*, 1990). Also, dry matter of Russet Burbank tubers was increased by about 23% by overexpressing one of the key enzymes involved in starch biosynthesis (Stark *et al.*, 1991, 1992). Some of these genetically engineered potato plants are being tested in the field (McCammon and Medley, 1990).

PROJECT STATUS: NEW

OBJECTIVES FOR 1993:

I. To clone complementary DNAs (cDNAs) that code for antifungal (permatins) proteins from potato. Oligo probes designed to conserved stretches of antifungal proteins will be used both in polymearse chain reaction and for cDNA library screening to isolate cDNAs.

II. To generate transgenic plants that are overexpressing antifungal proteins. This will be accomplished by transforming potato plants with an *Agrobacterium* based vector containing cDNA for antifungal protein fused to a constitutive (CaMV 35S) promoter.

The procedures that will be used in accomplishing these objectives are presented in Appendix I.

FUNDING REQUEST:

1993 Request: $ 7,000

The money requested in this project will be used for consumable supplies such as oligo primers, PCR and sequencing kits, restriction enzymes, radioisotopes, x-ray films, nitrocellulose filter and many routines chemicals and disposables. The P.I. will be submitting a seed grant proposal on the same project to Colorado Institute for Research in Biotechnology (CATI) to raise additional funds ($15,000) needed for the project. One of the requirements to submit a proposal to CATI is to obtain a minimum of 1:1 matching funds from university, federal or industry sources. The money requested in this project ($ 7, 000) together with $7,000 committed by the Dean of College of Natural Sciences and Chair, Department of Biology will be used as matching funds in applying for CATI Biotechnology Seed Grant. Majority of the equipment such as Thermal Cycler for polymerase chain reaction, hybridization incubators for screening are purchased from P.I. start-up funds. All the equipment that are needed to carry out the proposed work are available in the P.I's laboratory.

Appendix I (Procedures)

Isolation of Poly (A)$^+$ RNA: Total nucleic acids will be isolated according to Murray and Thompson (1980) with modifications (Jena *et al.*, 1989; Reddy *et al.*, 1990). Poly (A)$^+$ mRNA will be isolated using oligo d(T) cellulose column essentially as described (Sambrook *et al.*, 1989; Reddy *et al.*, 1990).

Isolation of cDNAs that code for anti-fungal proteins (permatins) from potato: Alignment of N-terminal amino acid sequence of antifungal proteins from different species indicate a stretch of highly conserved amino acid region (Vigers *et al.*, 1991). Degenerate oligonucleotide probe corresponding to the conserved amino acid stretch will be designed using codon preferences of potato. The oligoprobe will be used in two different approaches to isolate cDNAs corresponding to permatins. In one approach the oligo probe will be directly used to screen a potato cDNA library. In the second approach the oligo probe together with a oligo d(T) primer will be used in a polymerase chain reaction (PCR) to amplify antifungal protein (permatin) cDNA sequences from first strand cDNA. Synthesis of first strand cDNA will be carried out using oligo d(T) primer and reverse transcriptase (Jena *et al.*, 1989; Reddy and Poovaiah, 1990). Following the removal of RNA template, the first strand will be used in PCR with the oligo designed to conserved stretch of antifungal proteins (permatins) and oligo d(T). The amplified cDNA will be cloned and sequenced to confirm the identity and used to screen potato cDNA library to obtain full-length cDNA. The P.I. has considerable experience in using oligoprobes in PCR as well as screening cDNA libraries to isolate the desired genes. We have cloned a protein kinase and different types of cyclins using this approach.

Screening of the potato tuber cDNA library : For screening with oligos or polymerase chain reaction generated probes, a potato tuber library that we constructed will be used. We have successfully used this library to isolate a calmodulin cDNA from potato (Jena *et al.*, 1989). The cDNA library will be screened by plaque filter hybridization using oligonucleotide mixture labeled with 32P (Sambrook *et al.*, 1989). Prehybridization and hybridization will be carried our essentially as described by Hanks (1987). Filters will be washed with tetramethylammonium chloride according to wood *et al.*, (1985). Washing temperatures will be about 4^{o}C below the Td (Wood *et al.*, 1985). PCR generated probes will be labeled using random primers and hybridization and washings will be done as described earlier (Sambrook *et al.*,, 1989; Reddy *et al.*, 1990). Sequencing and analysis of the sequence will be done as described below.

Sequencing of clones: The cDNA inserts will be subcloned into pBluescript. The dsDNA template of pBluescript or single stranded phage DNA will be prepared and used as a template sequencing. Nested deletions will be made using exonuclease III according to Sambrook *et al.*, (1989). Sequencing will be determined by the dideoxynucleotide chain termination method using Sequenase (Sanger *et al.*, 1987). Sequence analysis will be performed by UWGCG or Eugene software.

Transformation of potato with sense constructs of antifungal proteins: Overexpression of antifungal proteins will be accomplished by generating transgenic plants containing antifungal protein cDNA in sense orientation fused to strong constitutive CaMV 35S promoter.

The expression vector pGA748 which contains neomycin phosphotransferase that confers resistance to kanamycin will be used to transform potato. pGA748 has seven unique restriction sites between the constitutive CaMV 35S promoter and the transcription terminator of the gene 7 (An *et al.*, 1988). The cDNA for antifungal protein will be cloned

into appropriate restriction sites in the multiple cloning region in sense orientation. The plasmid construct will be moved into *Agrobacterium tumefaciens* LB4404 by direct DNA transfer method (An, 1987). Potato (var. Russet Burbank) transformation will be performed according to a protocol that is routinely used in the P.I.'s laboratory. Leaf segments or internodes from axenically grown potato plants will be dipped in an *Agrobacterium* culture containing the desired pGA construct for 3 min, blotted on a sterile filter paper and transferred to callus induction medium (MS salts containing 1mg/L 2,4 D and 0.1mg/L kinetin) for 48 hours. Then the segments are transferred to shoot induction medium (MS salts supplements with 1mg/L thiamine HCl, 0.5mg/L nicotinic acid, 0.5mg/L pyridoxine HCl, 3 μM indole acetic acid, 5 μM zeatin riboside, 3% sucrose, 0.8% agar pH5.9) containing 50 μg/ml kanamycin and 500 μg/ml carbencillin. Kanamycin-resistant shoots will be rooted in MS medium. Several independent transgenic plants will be analyzed to identify the transformants that strongly express the introduced gene.

The level of the expression of introduced gene at the mRNA level will be monitored by northern analysis using RNA isolated from transgenic plants and strand specific RNA probes. Since our cDNA inserts are in a pBluescript vector which contains T3 and T7 promoters flanking the insert, we can generate strand specific RNA probes in an *in vitro* transcription system using T3 or T7 polymerase. Preparation of strand specific probes will be done according to Stratagene's instructions manual. The transgenic plants expressing the highest amount of expression of antifungal proteins will be tested for their resistance to various fungal phytopathogens of potato.

The P.I. has all the necessary expertise to accomplish the proposed goals. All the equipment, potato cDNA library, binary expression vectors with constitutive and tuber specific promoters that are needed for transfomation are available in the P.I.'s laboratory.

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1980 - 1984	Research Associate, Molecular Biology Unit School of Life Sciences Jawaharlal Nehru University New Delhi, India (Ph.D)
1985 - 1989	Postdoctoral Research Associate Laboratory of Plant Molecular Biology and Physiology Department of Horticulture and Landscape Architecture Washington State University, Pullman, WA
1989 - 1992	Staff Scientist Laboratory of Plant Molecular Biology and Physiology Department of Horticulture and Landscape Architecture Washington State University, Pullman, WA
1992 - present	Assistant Professor, Department of Biology Colorado State University, Fort Collins, CO

RESEARCH AREA: Plant Molecular Biology/Biochemistry

PUBLICATIONS:

Published over 75 scientific papers, book chapters, invited reviews and abstracts since 1982. Participated in fifteen national and international meetings. Participated in several practical courses including i) a three week (June 30th to July 21st, 1991) practical course on Molecular and Developmental Biology of Plants at Cold Spring Harbor Laboratories, New York, N.Y., ii) a two week (Nov. 25 - Dec. 8, 1990) genetic sequence computer analysis training course, at Molecular Biology Computer Research Resource Center, Dana-Farber Cancer Institute, Harvard Medical School, and iii) a week long course on essential techniques in gene manipulation (a practical course) from 21st-27th September, 1986 at University of Manchester Institute of Science and Technology, London.

Publications (Since 1987):

1. Poovaiah, B.W. and A.S.N. Reddy. (1993) Calcium and signal transduction in plants. CRC Cri. Rev. Plant Sci. (in press).

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