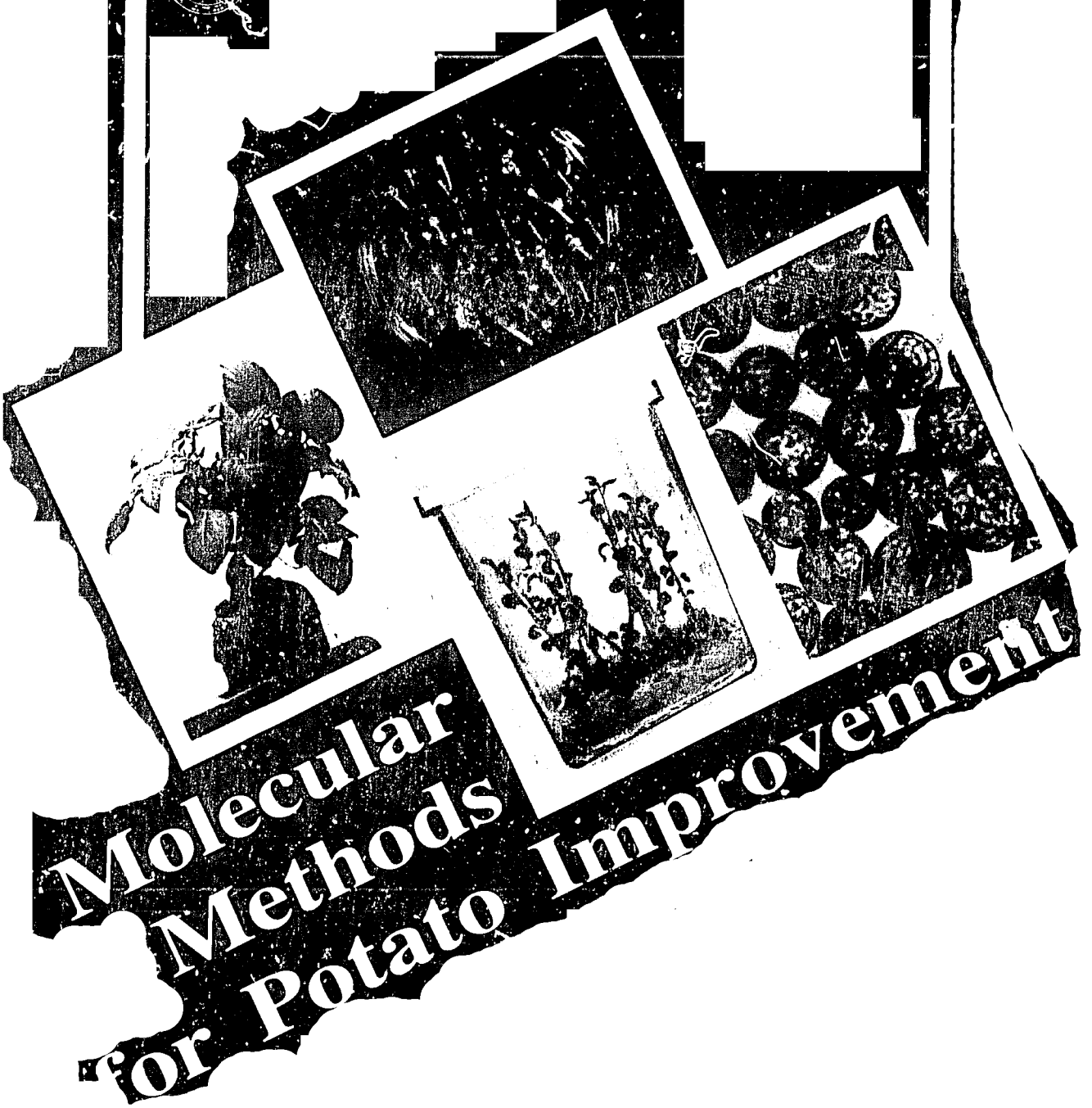
PN-ABJ-796

INTERNATIONAL POTATO CENTER (CIP)



Molecular Methods for Potato Improvement

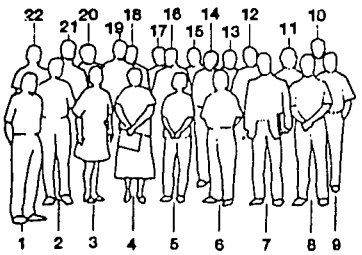
Report of the Planning Conference on

"Application of Molecular Techniques to Potato Germplasm Enhancement"

Held at the International Potato Center (CIP), March 5-9, 1990

1991

Participants at Planning Conference "Application of Molecular Techniques to Potato Germplasm Enhancement" (March 5-9, 1990).



1. Luis Lago Castro
   Programa de Papa
   Centro de Ingeniería Genética y Biotecnología
   CUBA

2. Kazuo Watanabe
   CIP

3. Meredith Bonierbale
   Department of Plant Breeding
   Cornell University
   U.S.A.

4. Lieselotte Schilde
   MNF Universität Tübigen
   GERMANY

5. Carla Montanelli
   Instituto Agronómico per L'Oltremare
   ITALY

6. Masachika Okamura
   Kirin Beer Brewery Co. Ltd.
   JAPAN

7. Marcelo Tejada
   Corporación Andina de Fomento (CAF)
   VENEZUELA

8. Joel Cohen
   Office of Agriculture
   Bureau of Science and Technology, AID
   U.S.A.

9. Ali Golmirzaie
   CIP

10. J.H. Dodds
    CIP

11. Primo Accatino
    CIP

12. Marc Van Montagu
    BELGIUM

13. Luis Destefano
    Department of Biochemistry
    Louisiana State University
    U.S.A.

14. Esra Galun
    Weisman Institute of Science
    ISRAEL

15. Ciro De Pace
    Universita Degli Studi
    Dipartimento di Agribiologia e Agrochimica
    ITALY

16. Claude Fauquet
    Washington University
    Department of Biology
    U.S.A.

17. Peter Gregory
    CIP

18. Richard L. Sawyer
    CIP

19. Charles Brown
    Irrigated Agriculture Research and Extension Center
    U.S.A.

20. Andrea Sonnino
    Dipartimento Fonti Alternative Rinmovabili Risparmio Energético
    ITALY

21. David Douches
    Michigan State University
    Crop & Soil Sciences Dept.
    U.S.A.

22. Wolf-Bernd Frommer
    IGR Berlin GMVA
    GERMANY

    Zózimo Huamán
    CIP

    Ian Robertson
    IITA/Univ. of Zimbabwe, Crop. Science Dept.
    ZIMBABWE

INTERNATIONAL POTATO CENTER (CIP)

Molecular Methods for Potato Improvement

Report of the Planning Conference on

"Application of Molecular Techniques to Potato Germplasm Enhancement"

Held at the International Potato Center (CIP). March 5-9, 1990

1991

Correct citation:

Book:

INTERNATIONAL POTATO CENTER (CIP). 1991. Molecular Methods for Potato Improvement. Report of the Planning Conference on "Application of Molecular Techniques to Potato Germplasm Enhancement", Lima, Peru, 5-9 March, 1990. Lima, CIP. 188 p.

Book Article:

DODDS, J.H. 1990. Application of Molecular Methods at CIP, Potato Improvement through Gene Identification and Gene Transfer. *In* Molecular Methods for Potato Improvement. Report of the Planning Conference on "Application of Molecular Techniques to Potato Germplasm Enhancement". pp. 45-48.

This publication was processed and printed
by the Information Sciences Department,
International Potato Center, January 1991.

Copies printed: 1 000

XIX-GR-E-01-O-1 000

ISBN-92-9060-148-5

PRESENTATION

At CIP, Thrust I research is directed at collection, characterization and utilization of potato and sweet potato genetic resources.

Over the last decade increasing use have been made of tissue culture and molecular methods in both the conservation and utilization of genetic resources.

The potato has become a model crop-plant for the application of molecular improvement techniques and the present planning conference clearly demonstrate that significant progress is being made on the direct agricultural application of these techniques.

The Planing Conference will help CIP to bring in world authorities from the scientific community to review objectives and procedures and help us to improve our work.

The meeting also allows a compilation of the current research status of this important research area. It is our intention that this proceedings serve as a permanent record of our objective and procedures. The recommendations imagine from the conference are indispensable to CIP and will be followed as closely as possible.

I would like to personally thank the participants in the planning conference for giving freely their time and energy.

Finally, I will like to thank the participants for submitting the manuscripts and to Patricia Chiroque, Ruth Delgado and Yoli Rivas for their efforts in preparing the final manuscripts. I do however accept responsibility for any errors that may have entered during the editing process .

John H. Dodds

Manager, Thrust I

ACKNOWLEDGEMENTS

Many CIP staff members and scientists in Advanced Research Institutes contributed to this "Planning Conference" by preparing drafts of sections or commenting on earlier versions of the manuscript.

Special thanks are given to J. Bemis, Y. Rivas, P. Chiroque, R. Delgado, K. Keenan, and A. Garcia-Pryce for their valuable help in the editing and production of these proceedings.

They served as editors, designed, typed and reviewed the entire manuscript and in this capacity provided invaluable guidance and comments during preparation of these proceedings.

The cover was designed by Cecilia Lafosse. Many photographs were provided by individual participants of the "Planning Conference".

John H. Dodds

"Molecular Methods for Potato Improvement"

CONTENTS

Objectives of the Planning Conference
P. Gregory 1
Recommendations 2

Session I

CIP's Mission and Molecular techniques for Germplasm Enhancements
Some Strategic Considerations for Future Impact
G. Scott 12
Potato Cell Engineering-Asymmetric Hybridization and Inter-Generic
Transfer of Disease Resistance.
M. Okamura 23
The Application of Molecular Techniques for Potato and Sweet Potato
Improvement in Latin America
L. Lago and S. Pérez 34
Application of Molecular Techniques for Potato Improvement in
Africa
I. Robertson 40

Session II

Application of Molecular Methods at CIP, Potato Improvement
Through Gene Identification and Gene Transfer.
J. H. Dodds 45
Use of Synthetic Genes to Enhance Nutritional Quality and Disease
Resistance in Plants: Application to Potato.
L. Destéfano, P. Nagpala, J. H. Dodds, and
J. M. Jaynes 49
Potato Gene Promoters and Their Applications
W. Frommer, U. Sonnewald, A. von Schaewen, S. Prat,
J. Sánchez-Serrano, J. Stockhaus, and L. Willmitzer 65
Coat Protein-Mediated Resistance: A New Type of Resistsance to
Control Plant Viruses
C. Fauquet and R. Beachy 72
Transformation of Potato (*Solanum tuberosum* L.) Leaf Disc Using
A. Tumefaciens-mediated Transfer of DNA Sequences
Coding for Lytic Peptides
C. de Pace, S. Trinca, R. Caccia, G. Scarascia, J. H. Dodds,
and J. M. Jaynes 85

Coat Protein-Mediated Resistance: A New Type of Resistsance to Control Plant Viruses
C. Fauquet and R. Beachy 72
Transformation of Potato (*Solanum tuberosum* L.) Leaf Disc Using *A. Tumefaciens*-mediated Transfer of DNA Sequences Coding for Lytic Peptides
C. de Pace, S. Trinca, R. Caccia, G. Scarascia, J. H. Dodds, and J. M. Jaynes 85
Biotechnology Biosafety and Field Testing: an Agency for International Development Perspective
J. Cohen 96

Session III

Protoplast Fusion for Potato-Cybrid Formation
E. Galun, D. Aviv, and A. Perl 105
Use of Protoplast Technology for Genome Transfer
L. Schilde and H. Ninnemann 112
Organelle Transfer (mitochondria) for the Production of Cybrid Potatoes and Their Possible use as TPS Parental Lines
A. Golmirzaie, J. Tenorio, and F. Serquén 121
Electrophoretic Characterization of Potato Germplasm at CIP
Z. Huamán 129
Present Status of Germplasm Enhancement at CIP and Application of Biotechnology
K. Watanabe 135
Induced Variation for Potato Improvement
A. Sonnino 141
Application of Restriction Fragment Length Polymorphisms and Genetic Mapping to Potato Breeding
M. Bonierbale, R. Plaisted, S. Tanksley 149
Species-specific DNA Probes in Introgression Breeding of Potato.
C. Brown, B. Hall, M. Hwang, A. Ager, B. McConaughy, and J. Helgeson 160
Use of RFLPs to Fingerprint North American Potato Cultivars.
D. Douches, R. Freyre, and K. Hicks 166

Session IV

The Andean Biotechnology Program (PAB) of the Andean Development Corporation (CAF)
M. Tejada 177

Objectives of the Planning Conference

Peter Gregory[1]

I would like to thank the visitors who have come to help us in this Planning Conference. Such conferences represent a very important strategy for timely review and planning of CIP's research activities. A very special aspect of the conferences, as exemplified here today, is that there is strong participation by the best available experts working in the developing and developed countries. During this Planning Conference on the "Application of Molecular Techniques to Potato Germplasm Enhancement" our objectives are:

1. To review CIP's previous work in this area and related work in other institutions.
2. To develop a set of recommendations which will serve for the next 3-5 years as guidelines for "in-house" research and for collaborations with scientists from around the world. These recommendations should map out what needs to be done, and, just as important, who should do it. Let us not take on work at CIP that others can do better elsewhere. Such recommendations are taken very seriously by CIP and only under very special circumstances are they not followed.

In this planning conference we must take into account not only scientific quality and productivity, but also how well the work has contributed, or will contribute, to CIP's impact on our partners, the NARS, and the people they serve. In fact, any activity that is unlikely to strengthen CIP's practical, agricultural impact must take a low priority for use of our resources, however scientifically interesting it might be. The impact of a given activity may be realized after as long as 10 or even 20 years, but the impact-orientation right from the start is what counts. For example, we, like CIMMYT and IRRI, have developed a strong interest in genome mapping. We hope that by using a genome map of the potato, we will eventually have the capability for selecting out and transferring genes for specific, agriculturally important traits. Thus we would make a major leap forward in our ability to specifically design and produce new potato genotypes without going through lengthy breeding processes. For many traits, it is unlikely that this will start to pay off for the NARS in less than 10 years. But this approach, which will involve large-scale collaboration with both the public and private sector, is justified in the sense that its enormous, potential, long-term impact is crystal clear.

In achieving the objectives of this Planning Conference, let us all be as open and as constructively critical as possible. In my opinion, we, in collaboration with many of you, have an excellent track record in the application of molecular techniques to potato germplasm enhancement, but I believe that during the next 4 days all of us here will work together to help make it even better.

[1]Dr. Peter Gregory, Director of Research, International Potato Center (CIP), Lima, Peru.

Recommendations

General Comments

These recommendations cover the areas of gene mapping, gene transfer by various vector systems, and the use of protoplast technologies, which are aimed at increasing the volume of improved genetic materials available to CIP's clients. The application of molecular techniques should increase efficiency in the production of improved germplasm and the incorporation of improved characters to CIP's breeding program this will thus speed up the availability of improved materials to National Programs.

GROUP 1
Recommendations on Genetic Mapping and Map Utilization

Introduction

A genetic map including DNA markers linked to genes of interest will be a valuable tool for germplasm enhancement and subsequent potato improvement. The greatest impact will probably come from the acceleration of breeding programs by reducing the number of sexual generations required to introgress and combine specific foreign genes with desirable genetic backgrounds.

This can be done because, once linkage is established between a mapped DNA marker(s) and a factor controlling a trait of interest, that marker or set of markers can be used to select individuals carrying only the effective piece(s) of chromosomes from the donor species, without genes that confer undesirable agronomic traits, such as long stolons or short day requirements.

It is our opinion that potential benefits of genetic mapping are sufficiently pertinent to CIP's mission to improve CIP's investment in further developing a genetic map of potato. CIP's strengths with regard to the exploitation of this technology are its capacity to identify germplasm sources that can be used to improve potato for priority traits, and to develop the appropriate populations required for the implementation of genetic mapping.

The first step in applying a genetic map toward a particular objective is to locate or 'tag' genes with respect to specific markers. These procedures rely on our ability to detect genetic differences among individuals in segregating progenies. We would like to stress the importance of

the use of appropriate populations (diploid, and segregating for one or more traits that we want to include in a cultivar) in RFLP-based gene mapping.

Genes of interest require certain prerequisites:

1. It should be demonstrated that statistically valid genetic differences exist among parental materials that are valuable for genetic analysis and breeding for a trait of interest. This means that there should be agreement (proven evidence) that screening is reliable and that the genetic source utilized is the best available source for improvement of cultivated material.

2. Target diploid population(s) segregating for high priority traits are needed for RFLP and phenotypic analysis. Analysis of these populations will provide the template map coordinates that guide marker-assisted selection in diverse populations at diploid (or tetraploid levels).

Marker-aided selection will not be cost-effective for single gene traits that are well expressed and readily identified with phenotypic screening procedures. e.g. PVY and PVX immunities.

These RFLP-based genetic maps will be most effective for improving potatoes for characteristics such as:

- multigene traits
- environmentally-sensitive traits
- hard-to-select traits
- quarantine pathogens or pests

The following pests and pathogens fall into this category:

i. bacterial wilt

ii. horizontal late blight resistance

iii. potato tuber moth

iv. *Erwinia* soft rot

v. PLRV

vi nematodes

Recommendations

1. Initiate in-house development of the RFLP map, fill gaps and saturate.

It is considered that creation of optimal populations may take further development. However, extension of the potato map may be commenced immediately with additional tomato probes which have high probability of targeting sparsely mapped regions. This will accomplish a

worthwhile advance of the potato gene map, and provide a good proving ground during the building of molecular expertise and opportunity for training of participants from NARS. Whether or not this should be done in-house at CIP will depend on the resources available.

In-house RFLP laboratory should be managed by a scientist with demonstrated ability in molecular techniques (i.e. post-doctoral).

2. Extensive evaluation of wild species is essential to identify better sources of resistance to *Pseudomonas*, *Erwinia* and *Phytophthora*. These should be brought into diploid populations to make RFLP analysis more efficient. Screening procedures must be improved for these traits along with research to uncover the mechanisms of each resistance.

3. CIP should provide in-house training in this technology to interested NARS, or contract a workshop to carry out this activity. Once the procedures are being used in Lima, CIP could play a role in coordinating a network among institutions working in potato RFLPs,

4. Characterize existing candidate populations at CIP (1) for bacterial wilt (partial resistance, *G. pallida* cyst nematode, root knot nematode resistance) (2) for frost resistance, PSTVd and PVX phenotypically, and, pending satisfactory results, proceed with RFLP analyses of traits.

5. Identify population(s) with traits that complement candidate populations existing at CIP. CIP should support proposals from outside institutions (contract) which include genetic mapping of proven high-priority traits if adequate facilities (laboratory productivity) and appropriate genetic material are demonstrated.

6. Long-term characterization of existing germplasm collection at CIP aided by RFLP, providing that basic "cluster-type" analysis has previously been done to formulate "core" or "representative" collection by the data base (passport data on morphology, isozyme and genetic attributes).

Priority Research Areas Availability Yes/No

Criteria	*Pseudomonas*	*Erwinia*	*Phytophthora*	PTM	PLRV
penetic					
Resistance	yes[a]	no	yes	yes	yes
Screening	yes	no	yes[a]	yes	yes
Population (2x)	yes[a]	no	no	yes	yes[a]
Best done CIP			Part		
Best contracted			Part		
Probability of success[b]	low	low	high	moderate	high

[a]Some reservations exist.
[b]The probability of success is dependent upon the current status of the basic criteria that must be met rather than the ability to apply RFLP-based mapping.

GROUP 2
Transformation Techniques and Field Testing

Introduction

We strongly suggest that CIP should continue to develop expertise in programs for the application of genetic engineering on potatoes. Due to its potential application for diverse sources of germplasm, the use of this technology is considered to be advantageous for solving specific problems not resolved through conventional procedures. The recommendations should be evaluated in context with the other biotechnological approaches and evaluated for each case.

Category I: General Recommendations

1. CIP should subscribe to international databases describing results from authorized field experiments and conduct a search within the public domain for constructs available globally of value to CIP and its client countries and then circulate this information to the NARS.
2. CIP should define procedures for a carefully selected set of genetic material so that regeneration and *Agrobacterium*-mediated transformation can be routinely performed for available constructs. CIP should demonstrate success in these procedures by inserting reporter genes in the selected material. If success is not achieved using tissue explants, then a protocol should be defined for protoplasts.
3. CIP should, in conjunction with the CG Secretariat, obtain legal advice regarding the use and acquisition of proprietary genetic material.
4. CIP should stimulate international exchange regarding the use and testing of transgenic potato plants. This could be accomplished through CIP's regional network of collaborators.
5. CIP should collaborate with national programs, where appropriate, to transfer constructs and transgenic plants.
6. CIP expertise should be developed, through training sabbaticals, to ensure that CIP scientists update their knowledge of advances in transformation technologies.
7. CIP should help NARS scientists to increase their knowledge of advances in transformation technologies.

Category II: Transformation Technologies (consult Table 1 and its discussion)

1. CIP should undertake validation and further development of the transformation procedures in those cases where plant engineering has reached the "prototype level."

2. When prototypes are not available and especially when CIP has a comparative advantage in either available germplasm or appropriate evaluation systems, CIP should participate through collaborative programs, with both advanced biotechnological laboratories and national programs.

3. CIP should encourage the development of basic research in transformation where it is needed.

Category III: Field Testing and Biosafety Considerations

1. CIP should ensure an appropriate "institutional biosafety" review of required field experiments. This could be accomplished by broadening the mandate of CIP's Germplasm and Quarantine Committee or by creating a new Biosafety Review Committee. In either case, it is recommended that CIP expand these efforts to include external specialists, as needed, to address environmental and safety considerations.

2. Testing procedures for transgenic material should include assurance that adequate certification for disease-free germplasm has been obtained.

3. CIP should acquire guidelines and information from existing regulatory programs that can be used to formulate field-testing guidelines for CIP.

4. CIP should formulate its own set of regulations, in conjunction with the CG Secretariat and national regulatory bodies, regarding the safe testing of the products of agricultural research.

5. CIP should undertake discussions with their regional or national collaborators and/or governments regarding the introduction and use of transformed plants in the field.

Potentials for impact of genetic engineering for potato

		To be contrated or encouraged by CIP / Could be done or coordinated by CIP			COMMENTS
		Basic Research done?*	Prototype made?*	Validation to be done?*	
Virus & Viroids	PVX-PVY	yes	yes	yes	(1)
	PLRV	yes	no	wait	(2)
	PSTV	In progress	no	wait	(3)
Insects	BT lepido	yes	yes	yes	(4)
	BT coleo	yes	yes	yes	(5)
Bacteria	Bacterial Wilt *Pseudomonas*	in progress	no	wait	(6)
Fungus	*Phytophthora*	in progress	no	wait	(7)
Nematodes	Nematodes	in progress	no	wait	(8)
Nutrition quality		in progress	no	wait	(9)

Note: This table is not in order of importance either for problems or feasibility

Table Comments

Potentials for impact of genetic engineering for potato

(*) Level of knowledge has been categorized in 3 levels: basic research, prototype, and validation.

- By "basic research" we mean that concepts for a particular genetically engineered trait have been established in host-pathogen models. For example, coat protein-mediated virus resistance as applied to different tobacco viruses is well established.

- By "prototype" we mean a sufficient accumulation of data on a particular trait in a particular plant. The coat protein mediated resistance has been proved to be extremely efficient for PVX and PVY in potato in field trials.

- By "validation" we mean the transfer of a particular technology to plant materials relevant for CIP. Coat protein-mediated resistance can be transferred to all potato cultivars where such resistance is needed.

(1) The PVX and the PVY coat protein genes have been inserted in potato and tested in field trials by for example, Monsanto Company. Three resistant lines, including an immune line, have been produced. The constructs exist and with appropriate agreement could be applied directly through *Agrobacterium*-mediated transformation. Many cultivars (commercial varieties and parental lines) can produce a sufficient number of shoots in order to select resistant lines. The validation of this genetic engineering will require expertise already existing at CIP, but will also require extension of CIP facilities. The technique may be applied on specific varieties or parental lines where natural resistance is not available or is too complicated to be integrated.

(2) The coat protein-mediated resistance for PLRV has not reached the "prototype" level but there is good evidence that resistance can be delivered by genetic engineering using this technique. Prototype development has not been undertaken by CIP.

(3) Though not included in the priority list of CIP, PSTV seems to be a critical problem, at least in the exchange and conservation of germplasm. The coat protein-mediated resistance strategy cannot be applied because it is a viroid. New basic concepts need to be established before any validation can be considered.

(4)(5) The *Bacillus thuringiensis* toxin strategy is well established and well documented, and several sources for obtaining the gene can be envisaged. Several prototypes have been made on potato for both lepidopterous and coleopterous insect pests. Constraints for CIP will be the same as those for PVX-PVY.

(6)(7) Natural and synthetic genes have been isolated (LSU in the U.S.A.) and are part of ongoing project with CIP. Some preliminary results are promising for the control of

phyto-bacterial diseases, as well as fungal diseases (by synthetic genes). More research is needed to sort out the precise effect of these synthetic peptides and their potential secondary effects on other organisms. CIP is participating in the transformation, regeneration and evaluation of transgenic plants expressing the inserted genes under controlled enviroments. Nevertheless, the true "prototype" has not yet been produced.

(8) The nematode resistance trait has been added to the CIP list of constraints because of its relationship with other diseases like bacterial wilt. Several approaches have been undertaken by many researchers, mainly on tomato, but no prototype has been established.

(9) Studies conducted in LSU (U.S.A.) led to the production of transgenic tobacco plants having an increased amount of total proteins. This interesting result needs to be confirmed and a prototype produced before CIP can validate this result.

Group 3
Recommendation on Cell Manipulations

1. Asymmetric Fusion

Fusion between x-irradiated protoplasts (donor) of one species and unirradiated protoplasts (recipients) of another species was successfully utilized in the past for the transfer of chloroplasts and/or mitochondria in order to produce cybrids composed of the nuclear genome of the recipient cultivar but organelles of the donor. This donor-recipient protoplast-fusion (DRPF) procedure was utilized for the establishment of breeding material in several crops (e.g. tobacco, potato, *Citrus* and several *Brassica* cultivars).

More recently the Asymmetric Fusion (AF) was developed. It is, in principal, similar to DRPF but differs in the details of the procedure and has different goals. The goals of AF are to transfer a small (but important in respect to crop improvement) fraction of the nuclear genome of the irradiated fusion-partner (donor) into the recipient cultivar. The dose of irradiation utilized in AF is commonly higher than in DRPF in order to desintegrate the donor chromosomes with the intent that DNA fragments from the donor will integrate into the nuclear genome of the recipient. Various methodologies were devoloped recently (e.g. RFLP identification, selective markers on specific donor chromosomes) to render the AF more efficient. The-Af derived plants used are then exposed to natural conditions (e.g. fields with a specific disease) to select, among these fusion-derived plants, individuals that show breeding advantages and are as similar as possible to the target cultivar.

We recommend that CIP utilize asymmetric fusion (AF) especially for the incorporation of nuclear components from wild *Solanum* species (donors) when such donors have been established for major potato diseases.

For example, since resistance to bacterial diseases (*Pseudomonas*, *Erwinia*) is a major aim at CIP, AF should be utilized to introduce the respective resistances into potato cultivars that already showed good performance in specific areas.

2. Cybridization

Cybridization by the DRPF was mentioned above and its applicability for conferring CMS in prospective "seed-parents" for F_1 hybrid TPS had a successful beginning at CIP.

We recommend that CIP continue to utilize cybridization by DRPF for both conferring CMS in certain breeding lines and cultivars (that showed promise as seed-parents for hybrid TPS) as well as to improve male fertility in other cultivars (that showed promise as pollen parents for hybrid TPS).

3. Somatic Hybridization

Somatic hybridization (SH) is a cell manipulation by which protoplasts of one fusion-partner are fused to protoplasts of another fusion partner and the heterofused cells are cultured in vitro and then regenerated into the respective somatic hybrid plants. This procedure has potential advantages for potato improvement, especially if diploid breeding lines that passed selection for various traits are available. SH among pairs of such breeding lines will cause the production of tetraploid somatic hybrids with good heterozygosity.

We recommend the use of (symmetric) somatic hybridization between selected diploid potato breeding lines having different quantitatively inherited trait.

4. Direct Transformation

Agrobacterium mediated transformation of potato in order to produce transgenic cultivars was discussed and recommended by Group 1. While this method, to introduce specific advantageous traits into target plants is the preferable means of genetic transformation, it is not applicable in all cases. The alternative is direct transformation (DT) of potato protoplasts with either appropiate expression vectors or with semi-homologous DNA fragments. The protoplast-to-plant system is well established for potato, and trasgenic plants were obtained by direct transformation with DNA constructs.

We recommend the use of potato protoplasts for genetic transformation. Such genetic material (i.e. DNA) may come in the form of expression vectors or isolated nuclear components of specific *Solanum* species. This DT technique is recommended in cases in which *Agrobacterium*-mediated transformation is not possible or inefficient.

Site of Research and Screening

1. We recommend that in-house capability in cell manipulation techniques should be established at CIP. However, most of the research should be treated as contracts or special research projects. We also recommend that especially when resistance to pathogens and pests have to be evaluated, field screening and evaluation should be carried out by CIP, and CIP breeders should be involved in these activities.

2. We believe that screening for diseases and pests and especially bacterial diseases is a bottle neck for breeding activities at CIP. We therefore recommend that CIP's capability in this area should be strengthened.

WORKING GROUPS TO FORMULATE RECOMMENDATIONS

March 8, 1990

GROUP 1: Genetic Mapping and Map Utilization

J. Cohen
Z. Huamán
K. Watanabe
M. Bonierbale
C. Brown
D. Douches

GROUP 2: Transformation Techniques and Field Testing

L. Lago Castro
I. Robertson
B. W. Frommer
C. Fauquet
L. Destéfano
M. Van Montagu
C. De Pace
C. Montanelli
H. El Nashaar
K. V. Raman
J. Bryan
M. Tejada

GROUP 3: Protoplast Techniques

M. Okamura
E. Galun
L. Schilde
A. Golmirzaie
A. Sonnino

CIP's Mission and Molecular Techniques for Germplasm Enhancement: Some Strategic Considerations for Future Impact

G. J. Scott[1]

Recent advances in biotechnology suggest that these techniques represent a potential avenue for accelerating and expanding the development of appropriate technologies for food production in the developing world (Buttel et al., 1985). With global population expected to reach 6.2 billion by the year 2000 (World Bank, 1989), the growing demand for food will certainly require a more rapid and increasingly diversified flow of technological innovations to meet these needs. Effective application of the latest techniques for germplasm enhancement to the problems of food production and utilization in developing countries, however, will depend to a great extent on strategies put in place to match technological capabilities with priority constraints and to target local, bilateral and multilateral efforts to areas which offer the greatest promise of impact. This paper suggests some key factors to consider when preparing a future strategy to capitalize on biotechnology for further improvement of potato production and utilization in developing countries. In so doing, it suggests locations and research topics that may be the most promising areas for collaboration between International Potato Center (CIP) scientists working with molecular techniques and those from other institutions with similar priorities in both developed and developing countries.

CIP's Mission

CIP was established in 1971 by means of an agreement with the government of Peru. CIP was first funded in 1972, when financing from the members of the Consultative Group for International Agricultural Research (CGIAR) became available to selected international agricultural research centers. Within the CGIAR system, CIP's mandate was initially defined as follows: "To develop, adapt and expand the research necessary for the technology to solve priority problems that limit potato production in developing countries" (TAC, 1989).[2] In simple terms, CIP's mission is to help produce the technology needed for potato (and, more recently, sweet potato) improvement, so as to make these crops available as low-cost foods in the developing world.

In assuming this responsibility, CIP scientists faced both a challenge and an opportunity. Although the potato originated in the highlands of South America and has been cultivated there

[1]Gregory J. Scott, Economist, Social Science Department, International Potato Center, P.O. Box 5969, Lima, Peru.
[2]In 1985, the Center's mandate was broadened to include sweet potatoes.

for over two millennia, the bulk of global potato production has been in the developed world for at least the last 100 years. Partly for that reason, CIP began its work without a substantial body of research on potato production and utilization in developing countries. The absence of this backlog of knowledge on potatoes in sub-tropical regions represents one important handicap to the generation of new technology for developing countries. The time-consuming nature of varietal generation for potatoes another. Even in developed countries a potato breeding program can take 6 to 7 years to develop a new variety and two decades or more to achieve impact (Sawyer, 1990). Consequently, while traditional breeding work progressed at CIP headquarters and in collaboration with national programs around the developing world, an interest developed simultaneously in alternative approaches to potato improvement so as to accelerate the process.

CIP scientists are fortunate in that the potato is a crop with an unusually rich and unexploited genetic background. According to Ross (1986), only a fraction of the genes available through wild and domesticated potato varieties has been utilized for breeding purposes. The World Potato Collection assembled by CIP therefore provides ample opportunity for innovative exploitation of a remarkably large and diverse quantity of genetic resources. Genes exist which can be combined with existing traits to enhance available germplasm. Furthermore, the most recent advances in molecular biology have been with plants that are part of the *Solanum* family, e.g. tobacco, which facilitates their applications to the potato when taking advantage of the potato's genetic diversity. The potential for future utilization as these techniques are improved and as the capacity to exploit them in developing countries expands raises strategic questions about their application to problems in specific regions and countries As input for this debate, the remainder of this paper briefly sketches trends in production and use of the potato in developing countries and then discusses priority constraints to potato improvement as seen through the opinion of national scientists.

Potato Production and Utilization

About 75% of the developing world's potatoes are produced in Asia (Table 1). Latin America and Africa harvest 17% and 8% respectively. Thus, although the potato is most often associated with the highlands of South America -- both as the center of origin for the crop and because of its present importance in the diet, economics, and culture in that region, the bulk of production is however elsewhere, in particular in China and India. These two countries alone account for two-thirds of all the potatoes grown in the developing world. It is also noteworthy that since the early 1960s a major share of the increase in production -- and roughly half the growth in area planted -- has been shared by these two countries.

Potatoes are grown in 94 developing countries of Asia, Africa and Latin America (Table 2).[3] Nevertheless, 27 of these 94 produce less than 10,000 tons per year. More importantly, perhaps, 28 countries produce more than 250,000 tons of potatoes annually and account for 90% of all the potatoes grown in the developing world (Table 3). These statistics suggest that in the immediate future, the demand for germplasm enhancement for potatoes through molecular biology techniques may be concentrated in relatively few countries rather than spread equally across the developing world for several reasons.

The great majority of potato producers in developing countries are located in those countries with the largest potato production. Farm-level pressure for improved potato technology will come from these growers. Most potato consumers in the developing world are in these same countries. The market for potatoes and the potential economic gains from the adoption of new technology therefore would appear to be greatest in these locations. Given the rapid expansion in area planted in most of these countries during the last two decades, they have a proven capability to take advantage of technological innovations. In other words, molecular techniques for germplasm enhancement should make improved varieties available in less time than traditional potato breeding programs. The growth in area planted suggests these countries have the ability to produce the quantities of planting material to effectively diffuse this type of technological innovation so as to facilitate impact.

Potatoes are grown under a tremendous variety of agroecological conditions. For the purposes of classification and at the risk of some oversimplification as most nations almost always contain an assortment of potato-producing zones, countries with the largest potato production can be grouped into one of the following, general agro-ecological categories : (1) temperate (i.e. long-days), e.g. Turkey, China, Chile, Argentina, Korea DPR, Korea R; (2) tropical lowland (i.e. cool winter; irrigated; <1,500 m) e.g. India, Bangladesh, Pakistan, Egypt, Algeria; and, (3) tropical highland (i.e. >1,500 m; mostly rainfed, but with areas under irrigation) e.g. Bolivia, Colombia, Mexico, Peru, Kenya, Rwanda, Nepal.[4]

The potato is a highly commercial crop in temperate and tropical lowland countries. Although farm families almost always eat at least some of what they produce, the overwhelming bulk of production (70-90%) is for sale. High yields for the crop under these growing conditions greatly facilitate this distribution pattern and allow for lucrative returns to potato production. In the tropical highlands, growers face low yields, geographic isolation from major population centers --and in sub-Saharan Africa-- the mushrooming demand for food in the countryside, all of which discourage greater sales.[5]

---

[3]There are 166 "states" in developing country category according to FAO statistics. FAO includes places like Hong Kong, Reunion as separate countries.
[4]For more information about types of potato producing countries see Horton (1987).
[5]For a detailed discussion of particular cases, see, e.g. Dürr and Lorenzl (1980) for Kenya; Scott (1985) for Peru and Scott (1988a) for Burundi, Rwanda and Zaire.

Although potatoes are often considered a poor man's food in much of Europe, they are an expensive, high-status consumption item in many developing countries.[6] Apart from potato producing households themselves, potato consumption in developing countries tends to be highest among higher income consumers. Moreover, in a number of detailed studies, the evidence strongly suggests that among poor households, potato consumption increases as incomes rise (Scott, 1985a; Scott, 1988b). Given the potato's high price in developing countries, only a tiny fraction of the crop is processed or transformed for industrial uses (Horton, 1988). Furthermore, perishability, bulkiness, the high ratio of transport costs to the final selling price and quarantine restrictions discourage international trade in potatoes. Only about 2% of annual production enters international commerce (Horton, 1987). The bulk of developing country potato output then is consumed fresh with a sizeable percentage also going to use as the farmer's own seed or for on-farm livestock feed (Horton, 1988). To summarize, the bulk of potato production in developing countries is found in Asia. Most of these potatoes are grown under temperate (i.e. long-day) or tropical lowland (i.e. cool winter, irrigated) conditions. Output is mostly for sale and to be used for human consumption within the country of production.

Constraints to Potato Improvement

Future expansion of potato production and use in developing countries will depend on the effectiveness of national and international efforts to overcome major agronomic, biological and socioeconomic constraints. Unfortunately, no comprehensive study of this topic from the developing country perspective exists. In view of this, and in order to capitalize on the abundance of location-specific knowledge in this field, a team of social and biological scientists at CIP conducted a survey of national program personnel to solicit their views about the importance of some 73 specific constraints grouped into 12 categories.[7] The following represents a discussion of the preliminary findings of that survey.

Initial analysis of the survey responses, i.e. simple averages of each of the responses for the 12 categories of constraints, indicate the prominent importance attached to marketing, planting material, seed storage, and viruses (Table 4). These findings appear to reflect the commercial orientation of growers as well as the high cost and timely availability of good quality (i.e. virus-free) seed. It also suggests efforts for germplasm enhancement would do well to consider postharvest --and not just production-- problems. It is noteworthy that national programs in all countries attach little importance to nematodes. Needless to say, these averages across all countries and categories tend to obscure important information about particular types of countries (e.g. those with large absolute quantities of potato production vs. those with meager amounts) or constraints.

[6]See Wolfe (1985) for a discussion of potato consumption in developing countries.
[7]The team was lead by Doug Horton, former head of the Social Science Dept. The results presented here are mostly the product of this work and that of his staff of assistants in the Social Science Dept.

Subsequent analysis of the survey results focused on the responses available for the 28 countries with the largest potato production (see Table 5).[8] These 28 countries produce 64.2 million tons of potatoes or 95% of the developing world's total. The available responses were from 16 of these 28 countries. These 16 countries produce 41.1 million tons of potatoes annually.

For this survey, marketing and storage were assigned particular importance by the national program respondents. This result seems logical in that those countries that produce considerable quantities of potatoes are likely to be more concerned about their storage and sale. Again, it points to the corcern in the developing world with postharvest as well as production constraints to future potato development.

Based on the work completed to date, it is useful to note the following. Little systematic information is available about the constraints to potato production and use in developing countries. CIP's survey represents a first attempt to fill this enormous and extremely critical void. Preliminary findings of this survey point to the potential usefulness of this information for decision-making about the future direction of research on the use of molecular biology techniques for germplasm enhancement. Scientists involved in this type of activity would do well, perhaps, to consider these findings as one criterion in priority setting. The importance of potato production in different types of countries is probably another that needs careful assessment in aiming for short-term impact. Those involved in the constraints, and production analysis work at CIP would welcome further inquiries from interested scientists.

References

Buttel, F. H., M. Kenney, and J. Kloppenburg, Jr. 1985. From Green Revolution to Bio-Revolution: Some Observations on the Changing Technological Basis of Economic Transformation in the Third World. Economic Development and Cultural Change, Vol. 34(1):31-56.

Dürr, G. and G. Lorenzl. 1980. Potato Production and Utilization in Kenya. International Potato Center. Lima, Peru. 133 p.

Horton, D. 1987. Potatoes: Production, Marketing and Progress for Developing. Countries. Westview Press. Boulder. 234 p.

Horton, D. 1988. Underground Crops: Long-Term Trends in Production of Roots and Tubers. Winrock International. Morrilton. 123 p.

Ross, H. 1986. Potato Breeding - Problems and Perspectives. Advances in Plant Breeding. Suppl. 13 to Zeitschrift Pflanzenzüchtung, Paul Parey, Berlin.

[8]One criticism of this perspective, however, is that it does not distinguish between regions within a given country. Hence, it distorts the location-specific importance of particular constraints. Future analysis of the survey findings will attempt to address this issue.

Sawyer, R. 1990. "Programing the Potato to Serve a Changing World". 2nd Triennial Meeting of the African Potato Association. Reduit, Mauritius. July 23-27, 1990.

Scott, G. 1985. Markets, Myths, and Middlemen: A Case Study of Potato Marketing in Central Peru. International Potato Center. Lima, Peru. 184 p.

Scott, G. 1988a. Potatoes in Central Africa: A. Survey of Burundi, Rwanda, and Zaire. International Potato Center. Lima, Peru. 159 p.

Scott, G. 1988b. Marketing Bangladesh's Potatoes: Present Patterns and Future Prospects. CIP-ADAB. Dhaka. 107p.

TAC. 1989. Report of the Third External Program Review of the International Potato Center (CIP). Rome. 89p.

Woolfe, J. 1987. The Potato in the Human Diet. International Potato Center and Cambridge University Press. Cambridge, England. 231 p.

World Bank. World Development Report 1989. Oxford University Press. Oxford. 251p.

Table 1. Potato production, area and yield in developing countries by regions, 1985-87.

Region	1961/63 Production[a] (000 t)	1961/63 Area[a] (ha)	1961/63 Yield[a] (t/ha)	1985/87 Production[a] (000 t)	1985/87 Area[a] (ha)	1985/87 Yield[a] (t/ha)
Africa[b]	1,749,906	237,481	7.4	5,244,649	641,357	8.2
	6.1%	6.7%	90.8%	7.8%	11.1%	69.7%
(Sub-Sahara)[c]	871,295	160,124	5.4	2,594,487	425,370	6.1
	3.0%	4.5%	67.0%	3.8%	7.4%	52.0%
Asia[d]	19,890,659	2,291,487	8.7	51,164,482	4,134,805	12.4
	69.0%	64.5%	106.9%	75.7%	71.8%	105.5%
(China)	12,908,267	1,434,349	9.0	27,611,621	2,448,067	11.3
	44.8%	40.4%	110.8%	40.9%	42.5%	96.2%
Latin America[e]	7,181,023	1,021,060	7.0	11,180,713	986,620	11.3
	24.9%	28.8%	86.6%	16.5%	17.1%	96.6%
Total	28,821,588	3,550,027	8.1	67,589,844	5,762,782	11.7

Source: FAO Statistics.
[a] Percent of total production/area planted/average yield for all developing countries.
[b] Africa not including South Africa.
[c] Africa -[Morocco, Algeria, Tunisia, Egypt, Libya]-[South Africa].
[d] Asia - [Israel, Japan] + Oceania -[Australia, New Zealand].
[e] North and Central America + South America - [Canada, USA].

Table 2. Distribution of developing countries by volume of production, 1983-85.[a]

Potato Production	Number of Countries			
	Africa	Latin America	Asia	Total
0 or no information	22	22	28	72
< 10,000 t	9	8	10	27
< 50,000 t	9	6	7	22
< 250,000 t	7	2	8	17
< 250,000 t	7	9	12	28
Total	54	47	65	166

Source: FAO Statistics.
[a]*FAO* categorizes all states (eg. Hong Kong and Reunion) on an equal basis as developing countries. These figures reflect that categorization.

Table 3. Twenty eight developing countries with the largest potato production.

Country	Production (000 t) 1961-63	1985-87	Increase (%)[a]
1 China	12,908	27,594	114
2 India	2,844	12,874	353
3 Turkey	1,498	4,130	176
4 Colombia	665	2,300	246
5 Brazil	1,127	2,103	87
6 Argentina	1,570	2,060	31
7 Korea Dpr	720	1,945	170
8 Iran	317	1,853	485
9 Peru	1445	1,744	21
10 Egypt	389	1,485	282
11 Bangladesh	347	1,171	237
12 Mexico	366	964	164
13 Algeria	234	892	282
14 Chile	819	847	4
15 Morocco	197	789	300
16 Bolivia	531	754	42
17 Pakistan	119	593	397
18 Korea Rep	422	489	16
19 Nepal	182	476	162
20 Indonesia	60	414	589
21 Ecuador	284	383	35
22 Ethiopia	132	370	181
23 Viet Nam	45	348	673
24 Kenya	194	337	74
25 Malawi	62	312	403
26 Cuba	96	284	197
27 Afghanistan	67	284	325
28 Syria	32	278	772

Source: FAO Statistics.
[a]1985-87 vs 1961-63.

Table 4. Constraints to potato production and use in developing countries.[a]

Category of Contraint	Average	Standard Deviation
Marketing	1.72	0.73
Planting Material	1.59	0.83
Seed storage	1.33	0.72
Virus diseases	1.17	0.68
Bacterial diseases	1.14	0.64
Fungal diseases	1.06	0.47
Consumer storage	1.05	0.85
Demand	0.92	0.59
Environment	0.71	0.42
Insects	0.67	0.35
Nematodes	0.58	0.60
Varieties	0.46	0.51

Source: Constraints Survey.

Key: 0 = Constraints not present or no informed.
1 = Constraint of minor importance.
2 = Constraint important.
3 = Constraint very important.

[a]These are simple arithmetic averages with corresponding standard deviations, based on 44 valid survey forms completed representing 43 developing countries. See text and CIP 1989 Annual Report (Thrust X) for additional information.

Table 5. Constraints to potato production (averages weighted by production) in the 28 countries with the largest potato production.

Group	Specific	Averages	
Marketing	High marketing costs or profits	0.75	1.13
Marketing	Transportation problems	1.02	
Marketing	High handling losses	1.11	
Marketing	Unstable supplies and prices	1.60	
Consumer storage	High storage costs	0.44	0.88
Consumer storage	Rodents (consumer storage)	0.45	
Consumer storage	Insects (consumer storage)	0.86	
Consumer storage	Shriveling (consumer storage)	0.88	
Consumer storage	Diseases (rots-consumer storage)	1.00	
Consumer storage	Poor store design (consumer storage)	1.07	
Consumer storage	Insufficient storage capacity (consumer storage)	1.11	
Consumer storage	Sprouting (consumer storage)	1.11	
Seed storage	Shriveling (seed storage)	0.36	0.86
Seed storage	High storage costs (seed storage)	0.52	
Seed storage	Insects (seed storage)	0.62	
Seed storage	Rodents (seed storage)	0.80	
Seed storage	Diseases (rots- seed storage)	1.06	
Seed storage	Sprouting (seed storage)	1.11	
Seed storage	Insufficient storage capacity (seed storage)	1.20	
Seed storage	Poor store design (seed storage)	1.22	
Environment	Soil salinity	0.02	0.71
Environment	Low light energy (cloudiness)	0.17	
Environment	Low soil pH	0.20	
Environment	Aluminum toxicity	0.35	
Environment	Excess soil moisture	0.43	
Environment	Heat	0.46	
Environment	Poor soil structure	0.95	
Environment	Weeds	1.13	
Environment	Cold, frost or hail	1.20	
Environment	Drought	1.26	
Environment	Low soil fertility	1.58	
Demand	Potato not in traditional diet	0.06	0.45
Demand	Potato not available	0.09	
Demand	People don't like to eat potatoes	0.10	
Demand	Price too high	0.45	
Demand	Lack of variety with good processing quality	0.52	
Demand	Lack of a variety with good eating qualities	0.84	
Demand	Lack of processed products	1.09	
Insects and mites	Leaf miner fly	0.08	0.33
Insects and mites	Leaf hoppers	0.09	
Insects and mites	Thrips	0.09	
Insects and mites	Andean tuber weevil	0.10	
Insects and mites	Tuber moth	0.10	

Group	Specific		Averages
Insects and mites	Aphids	0.23	
Insects and mites	Mealy bugs	0.31	
Insects and mites	Mites	0.86	
Insects and mites	Cutworms	1.07	
Virus diseases	Mycoplasms	0.13	0.17
Virus diseases	Leaf roll virus (PLRV)	0.13	
Virus diseases	Virus Y (PVY)	0.23	
Planting material	Scarcity	0.07	0.15
Planting material	Poor physiological condition	0.11	
Planting material	High cost	0.16	
Planting material	Poor health	0.25	
Varieties	Dormancy too long	0.05	0.11
Varieties	Poor market/consumer acceptance	0.08	
Varieties	Not suited for livestock feeding	0.09	
Varieties	Too late maturing	0.11	
Varieties	Not suited for processing	0.11	
Varieties	Dormancy too short	0.18	
Varieties	Too early maturing	0.20	
Fungal diseases	Wart	0.04	0.09
Fungal diseases	Rhizoctonia stem canker or black scurf	0.06	
Fungal diseases	Tuber dry rot	0.09	
Fungal diseases	Early blight	0.10	
Fungal diseases	Late blight	0.17	
Bacterial diseases	Bacterial wilt	0.08	0.09
Bacterial diseases	Soft rot of tubers	0.09	
Bacterial diseases	Black leg	0.10	
Nematodes	Cyst nematode	0.05	0.05
Nematodes	Root knot nematode	0.05	

Source: Constraints Survey.

Potato Cell Engineering-Asymmetric Hibridization And Inter-Generic Transfer Of Disease Resistance

M. Okamura[1]

Profile

The potato (*Solanum tuberosum* L.) is a tetraploid, highly heterozygous, and a vegetatively propagated crop. Polyploidy and sterility are serious constraints in potato breeding through sexual crossing. The outcome of crosses between tetraploid parental lines is often unpredictable. Genotypes superior for a sufficient number of desired characters are extremely rare in such progenies. Spontaneous somatic mutations occur in potatoes with quite low frequency. Potato plants can be regenerated from organized tissue, cell aggregates, and from protoplasts. Plants regenerated from protoplasts are characterized by an outburst of variations. Protoclonal variation can give us the chance to recover cultivars e.g. cultivars that obtain disease resistance while retaining their desirable, agronomically-important qualities. Protoplast systems show potential for the improvement of potato, namely the production of somatic hybrid plants mediated by protoplast fusion. Somatic fusions may provide a means by which traits from sexually incompatible species can be incorporated into potato. Reasonably efficient plant regeneration systems from protoplasts are required for these purposes (Okamura, 1988; Okamura, 1989)

Somaclonal Variation in Potato

Efficient plant regeneration from protoplasts of potato, some Solanum and Lycopersicon species

The plant species used for isolating protoplasts and for regenerating plants are listed in Table 1. Mesophyll protoplasts were isolated from the leaves of axenic shoot cultures growing on Murashige and Skoog medium without growth regulator. Prior to enzyme treatment, the leaves were cut into small fragments (1 mm width) and cultured in PEC (pre enzyme culture) medium in the dark at 25C for 16-40 hours. PEC medium is a modified MS medium where NH4NO3 concentration is reduced to 300 mg/l and supplemented with 1 mg/l NAA and 1 mg/l BA. This PEC treatment was found to promote cell division and subsequent colony formation.

For example, plating efficiencies of *S. tuberosum* and *S. demissum* protoplasts were doubled by this treatment. It is apparent from several reports that the physiological status of the protoplast-donor tissue or cells has profound influence on protoplast viability. This PEC

[1]Masachika Okamura. Plant Laboratory, KIRIN Brewery Co., LTD., Tochigi, Japan

treatment of the leaves may make uniform the physiological status of the mesophyll cells and/or may trigger a change in the behavior of cells from inability to ability of division (Okamura, 1989)

Protoplasts were isolated enzymatically from the PEC-treated leaves, and cultured in protoplast medium. After 5-10 days of culture, protoplast-derived colonies were transferred into colony medium and subcultured weekly in the same medium. The developing calli were transferred onto shoot-induction medium. Regenerated shoots were transplanted to rooting medium. Whole plants were regenerated from protoplasts of the species in Table 1 from 9 to 18 weeks after protoplast isolation (Table 1) (Okamura, M., and Momose, 1989). Plant regeneration from protoplasts of these species was performed by a protocol developed by Okamura (Okamura M., 1988a).

Somaclonal variation in plants regenerated from potato protoplasts

The frequency of genetic changes is significantly higher for somaclonal variation than observed for spontaneous genetic changes. Regenerated plants are derived from single cells; hence, mosaics occur at very low frequency. The plant regeneration step acts as a sieve that eliminates most deleterious genetic changes. These features make somaclonal variation attractive for crop improvement.

We have produced more than 200 lines of protoclone (protoplast-derived plants) from mesophyll protoplasts of potato line KRN0928, and examined variations. Protoclonal variation was observed in leaf morphology, flower color, tuber shape, etc. For example, among 214 lines of protoclone tested in the field, have deep purple flowers 7% and 2% have white flowers although the original KRN 0928 has pale purple flowers.

Variation in agronomically useful traits in potato protoclones was first reported by Shepard et al. They examined over 10,000 clones regenerated from protoplasts of the cultivar "Russet Burbank," and found variations in growth habits, tuber shape and size, maturation date, disease resistance and so on.

In addition to variations in agronomical characters, we have found a variant that is amenable to protoplast culture among *Solanum phureja* protoclones (Okamura and Momose, 1989). Although plating efficiency and regeneration efficiency of protoplasts of *S.phureja* line KRN01 are very low, protoclone line 02 showed 4 times higher plating frecuency and 5 times higher regeneration frequency than those of the original plant (Table 1).

Somaclonal variation is useful where specific modifications are required in otherwise well-adapted cultivars, and will be more powerful if combined with cell selection techniques.

Somatic Hybridization in Potato

Intraspecific, interspecific and intergeneric somatic hybrid plants in potato

Useful traits from sexually incompatible species can be incorporated into potato by somatic hybridization. Another important aspect of potato somatic hybridization is intraspecific protoplast fusion in the analytic-synthetic breeding scheme proposed by Wenzel et al., 1979. In that scheme, monohaploids of divergent origin carrying maior genes for resistance to four different pathogens are bred to tetraploids by polyploidization, crossing and somatic hybridization. Production of potato monohaploids through prickle pollination and protoplast fusion of these monohaploids was reported by Uijtewal et.al (1987). But, for the full realization of the analytic-synthetic breeding schemes, problems such as genotype dependency in anther and protoplast culture, evaluation of agronomical characters in monohaploids and so on should be overcome. Somatic hybridization of selected inter-dihaploids (heterogeneous) carrying different useful characters would be a practical method for maximizing heterozygosity in the resulting tetraploid.

The potato has been improved by the introgression of characters from the wild relatives of the cultivated potato using sexual crossing. However, as useful as these wild relatives have been in potato breeding, a vast reserve of additional genetic resources that exists in the wild species of *Solanum* and *Lycopersicon* remains unexploited because of the species sexual incompatibilities with the cultivated potato. In potato, many interspecific and some intergeneric somatic hybridizations have been reported so far. Somatic hybrids between potato and diploid wild species *S. brevidens*, which has potentially useful characters, have been regenerated (Austin et al., 1985; Austin et al., 1988; Fish et al., 1988; Irikura et al., 1987) and the hybrids have proved to be fertile and resistant against PLRV and tuber soft rot (Austin et al., 1988). Somatic hybrid plants have been produced also between potato and *S. chacoense*, which combined chloroplasts from *S. chacoense* and mitochondria originating from *S. tuberosum*. This shows that unique combinations of genetic material can be produced somatically.

At the Plant Laboratory of KIRIN Brewery, have succeeded in the production of both interspecific and intergeneric somatic hybrid plants in potato.

Regeneration of somatic hybrid plants between potato and S.photeinocarpum

Solanum photeinocarpum, a wild species in *Solanum*, has resistance to late blight, but is cannot be crossed sexually with potato. More than 20 lines of somatic hybrid plants between this wild species and potato have been produced by protoplast fusion. The hybrids differ morphologically from other parent.

Hybridity was verified by analysis of aspartate aminotransferase isozymes. All of the somatic hybrid plants have specific isozyme bands for both potato and the wild *Solanum*. The most vigorous hybrid plant has 72 chromosomes (the sum of both parents). We will examine other characters of the hybrids e.g. disease resistance, fertility, etc. (Momose and Okamura, 1989). This work was supported partially by MAFF (Ministry of Agriculture, Forestry and Fishery of Japan).

Regeneration and evaluation of somatic hybrid plants between potato and a wild tomato (Lycopersicon pimpinellifolium)

Intergeneric somatic transfer of disease resistance. "Pomato", somatic hybrids between potato and tomato, demonstrated a possibility of overcoming the crossing barrier between *Solanum* and *Lycopersicon*. *Lycopersicon pimpinellifolium* (Jusl.) Mill. (2n=24), a wild species of tomato, has important traits for potato breeding such as resistance to bacterial wilt, soft rot and high temperature, but is cannot be crossed sexually with potato. We have produced somatic hybrid plants between potato and the wild tomato (Okamura, 1988a, Okamura, 1989). Mesophyll protoplasts of both species were isolated from in vitro shoot cultures and electrofused. The fusion-treated protoplasts containing heterokaryons were cultured in a series of media mentioned above, and 134 lines of somatic hybrid plants were regenerated (Fig. 1). Hybridity was verified by morphological characteristics and malate dehydrogenase isozyme analysis. Many of the somatic hybrids grew vigorously in the field and flowered. Some lines set berries with many seeds. The morphology of leaves, flowers, berries and tubers varied from one line to another. The outburst of variations was partly due to the difference in chromosome numbers between the somatic hybrid lines (2n=58, 65, 72, >100, etc).

Somaclonal variation seems to be a general phenomenon in protoplast-derived plants of at least potato and tomato. It is not surprising that somaclonal variation has been detected in plants regenerated from fused protoplasts. A certain somatic hybrid line has prickles on each leaf, although neither potato nor the wild tomato has prickles

In the disease resistant test of the acclimatized plant, some lines of somatic hybrids were found to be resistant against bacterial wilt and soft rot (Fig. 2). A bacterial suspension was added to the cut surface of root (bacterial wilt) or leaf (soft rot) of the acclimatized plant. As for bacterial wilt, some somatic hybrid lines showed delayed expression of the disease symtom. In soft rot, control plants became soft and rotted within 7 days after infection. However, somatic hybrid lines out of 30 grew normally to mature plants. This is the first demonstration of intergeneric somatic transfer of disease resistance in potato (Okamura, 1989)

Genetic modification of somatic hybrid plants by cell engineering

Protocloning and X-ray irradiation of somatic hybrid plants between potato and the wild tomato. In order to eliminate undesirable genes from the wild tomato. That still exist in the somatic hybrid plants, backcross of potato to somatic hybrids is required. However, backcross is difficult when the fertility of the somatic hybrid is low. Besides backcrossing, other means to modify genetic constitution of somatic hybrids include cell engineering techniques such as protocloning (i.e. the production of somaclonal variants of the somatic hybrids), irradiation with X-rays or gamma rays of the protoplasts of the somatic hybrids, back-fusion of the somatic hybrids with potato (somatic backcross), etc. (Okamura, 1989).

Mesophyll protoplasts of the disease resistant somatic hybrid were isolated, cultured as described above and 208 protoclones were regenerated. All the protoclones were confirmed as

somatic hybrids by malate dehydrogenase isozyme analysis. Among those protoclones, some lines showed higher vigor in the field as compared with the protoplast-mother somatic hybrid. One protoclone line proved to have ability to set seeds, thought the protoplast-mother somatic hybrid had no seed set ability.

Irradiation of protoplasts isolated from the disease-resistant somatic hybrid was also tried. Mesophyll protoplasts of the somatic hybrid were exposed to X-irradiation (5kR, dose rate; 380 R/min), and cultured as described above. Out of 3,000 colonies transferred onto shoot-induction medium, only two colonies produced shoot, and two lines of somatic hybrid origin were regenerated from X-irradiated protoplasts. Among these two lines, one had an intermediate-type morphology of both parents (potato and the wild tomato), and the other had gross-aberrant morphology. Isozyme analysis for the shoot on aspartate aminotransferase (AAT) and peroxidase (PRX) showed that these two lines had a combination of bands of the parental species (Okamura, 1989; Okamura and Momose, 1989).

Back-fusion (somatic backcross) and the production of asymmetric somatic hybrid plants carrying the complete genome of potato plus a few chromosomes or chromosome fragments from the wild tomato.

Another way to modify genetic constitution of the hybrids would be back-fusion (somatic backcross). We have tried back-fusion of the disease-resistant somatic hybrid with the potato, and succeeded in the production of asymmetric somatic hybrid plants (partial hybrids) (Fig. 3).

Somatic hybrid plant line PT31 (a disease-resistant somatic hybrid between potato and the wild tomato) and *Solanum tuberosum* line KRN0928 (a potato line which is highly fertile and amenable to cell culture) were selected as materials for the back-fusion experiment so mesophyll protoplasts of somatic hybrid line PT31 were exposed to X-irradiation (30 kR), and electrically fused with unirradiated, iodoacetamide(IOA)-treated (10mM IOA, 4 C, 15 min.) protoplasts of potato. This double inactivation scheme, iodoacetamide treatment of one parent in combination with irradiation of the other, enabled us to obtain heterofusion-derived clones. High doses of X-irradiation (30 kR) promotes chromosome elimination of the irradiated parent.

More than 150 colonies derived from back-fusion-treated protoplasts produced shoots. Finally, 197 lines of back-fusion derived plants were obtained. These regenerants were classified by their morphology into 3 types; potato-type, intermediate-type, and gross-aberrent-type. Twenty-two lines of regenerants were selected at random from potato-type and intermediate-type plants, and their isozymes were analyzed.

Intermediate-type plants tested had AAT and PRX isozymes from both parental species (potato and the wild tomato). To the contrary, all of the potato-type plants tested had lost AAT isozyme bands specific to the wild tomato, while retaining AAT bands from potato. However, peroxidase (PRX) isozyme analysis of these potato-type plant lines showed that 5 lines out of 11 (45%) had a combination of bands of both parental species (potato and the wild tomato). The

PRX band detected only in the wild tomato (not in the potato) could not be found in protoclones of the potato. Chromosome analysis of potato-type somatic hybrid plant lines demonstrated that all the lines tested had a smaller number of chromosomes (2n=44, 52, 62, 76, 84) than the sum of both fusion parents (potato;2n=48, PT31;2n=60). The results indicate that at least 5 potato-type plants derived from back-fusion are asymmetric hybrids (partial hybrids) carrying the potato genome plus a few chromosomes or a chromosome fragment from the wild tomato (Okamura, 1989; Okamura and Momose, 1989).

Microtubers produced by potato-type asymmetric hybrids had a similar morphology to those of field grown potato. Among these potato-type asymmetric hybrids, line BF 13 grew vigorously in the field and produced tubers very similar to potato tubers (Fig. 4). We will examine hybridity of other back-fusion regenerants, and test their disease resistance, fertility, etc., (Okamura, 1989).

Nuclear (gene cluster) donor-recipient protoplast fusion

The control of nuclear gene cluster incorporation from donor to recipient in asymmetric hybridization Despite many reports on asymmetric hybridization, there have been few examples of successful introduction of agronomically important, nuclear-encoded characteristics from donor plants (e.g. wild species) into recipient plants (e.g. cultivars). (Here the term asymmetric refers to the nuclear constitution of hybrids.) The problem is the low efficiency of the screening for the useful hybrids. Since the possibility of the eliminating of desired genes of donor plants becomes higher as the amount of genes introduced into recipients decreases, a vast number of hybrids are required to screen out for the useful traits. Even when a useful asymmetric hybrid is obtained, a second somatic hybridization is necessary to transfer the useful trait to other cultivars or species if the fertility of the hybrids is low, and a vast number of asymmetric hybrids are again required to be screened out for the useful trait. Consequently it is very important in asymmetric hybridization to efficiently incorporate only those genes that are responsible for agronomically desirable traits.

One solution to the problem is given by the use of the protoplast fusion technique together with recombinant DNA technique (Okamura, 1988). Recent progress in the genetic engineering of higher plants now allows the introduction of a selectable dominant marker gene such as to antibiotic resistance into the genome of the donor to establish a set of donor lines with the marker gene incorporated into different positions on the genome. After the asymmetric hybridization of the recipient with each of the donor lines irradiated with X-ray or gamma-ray, a set of asymmetric hybrids carrying the marker gene can be easily selected on an appropriate selectable medium. By evaluating the desired agronomical trait of asymmetric hybrid plants regenerated from these hybrids, we would be able to identify particular donor lines ("linkage-donor" lines) in which the desired agronomical trait is linked with the selectable marker from the original set of donor lines. "Linked-donor" candidates can be selected also by linkage analysis on the marker gene and the target gene cluster. Once linkage-donor lines are obtained, we can transfer the desired trait from these donors to any recipient through asymmetric hybridization, which should be called "nuclear

(gene cluster) donor-recipient protoplast fusion" (Fig. 5), since it would allow effective in vitro selection of asymmetric hybrids that incorporate the desired trait by the selectable marker.

As is shown in the examples above, somatic crossing mediated by protoplast fusion is a noteworthy system for transferring several genes or unidentified genes of interest from wild species into cultivars, and will play an important role in plant breeding.

Acknowledgments

The autor wisher to thank Mr. Y. Kagami, Mr. T. Kato and Dr. K. Ohmori for their useful suggestions and M. Momose, Y. Yokoyama, K. Mori, F. Arima, M. Yada, M. Suzuki, and N. Okamura for the help in various phases of the research.

References

Austin, S., M. A. Bare, and J. P. Helgeson. 1985. Transfer of resistance to potato leaf roll virus from *Solanum brevidens* into *Solanum tuberosum* by somatic fusion. Plant Science 39:75-82.

Austin, S., E. Lojkowska, M. K. Ehlenfeldt, A. Kelman, and J. P. Helgeson. 1988. Fertile interspecific somatic hybrids of *Solanum*: A noble source of resistance to *Erwinia* soft rot phytopathology 78:1216-1220.

Fish, N., A. Karp, and MGK Jones. 1988. Production of somatic hybrids 12 by electrofusion in *Solanum*. Theor. Appl. Genet. 76:260-266.

Irikura Y., Y. Ohhashi, Y. Ohkawa. 1987. Production of somatic hybrid plant between *Solanum tuberosum* and *Solanum brevidens* by protoplast fusion (in Japanese). Japanese Journal of Breeding 37, suppl. 1:114-115.

Momose M., and M. Okamura. 1989. Regeneration of somatic hybrid plants between potato and *S. photeinocarpum* (in Japanese). Japanese Journal of Breeding 39, suppl. 1:92-93.

Okamura, M. 1988. Regeneration and evaluation of somatic hybrid plants between *Solanum tuberosum* and *Lycopersicon pimpinellifolium*. *In* K. J. Puite et al.(eds.) Progress in Plant Protoplast Research. Kluwer Academic Publishers, Dordrecht. pp. 213-214.

Okamura, M. 1988. Plant breeding through cell fusion (in Japanese). Bio Science & Industry, Bioindustry Development Center, Shinbashi, Tokyo, Japan 46:12-18

Okamura, M. 1989. Cell engineering in potato (in Japanese). The Tissue Culture, 15:344-348, The New Science Co., Kanda, Japan.

Okamura, M., and M. Momose. 1989. Genetic modification of somatic hybrid plants by cell engineering (in Japanese). *In*: Proceedings of the 11th Plant Tissue Culture Symposium, Okayama, Japan. p. 234.

Okamura. M., and M. Momose. 1989. Efficient plant regenerarion from protoplasts of potato and several wild *Solanum* species (in Japanese). Japanese Journal of Breeding 39 Suppl. 1:90-91.

Uijtewall, B. A., E. Posthumus, LCJM. Suurs, E. Jacobsen, and JGTh Hermsen. 1987. Relative performance of monohaploid potato clones and their diploid parents at plant level and after protoplast isolation and subsequent fusion. Theor. Appl. Genet. 74:451-458.

Wenzel, G., O. Schieder, T. Przewozny, SK. Sopory, and G. Melchers. 1979. Comparison of single cell culture derived *Solanum tuberosum* L. plants and a model for the application in breeding progams. Theor. Appl. Genet. 55:49-55.

Table 1. Efficiency of plant regeneration from protoplasts of some *Solanum* and *Lycopersicon* species.

Plant species	Lines	Plating efficiency (%)[a]1	Regeneration efficiency (%)[b]2
Solanum tuberosum	KRN0810	12	60
	KRN0928	20	80
	KRN0929	20	80
Solanum phureja	KRN01	5	5
protoclone	02	20	25
Solanum nigrum	70	90	
Solanum photeinocarpum	70	90	
Solanum demissum	50	-	
Lycopersicon pimpinellifolium	30	20	

[a]1:defined of the initial number of plated protoplast as a percentage of the number of colonies
[b]2:defined as a percentage of the number of shoot-forming colonies of the number of colonies plated in the regeneration medium.

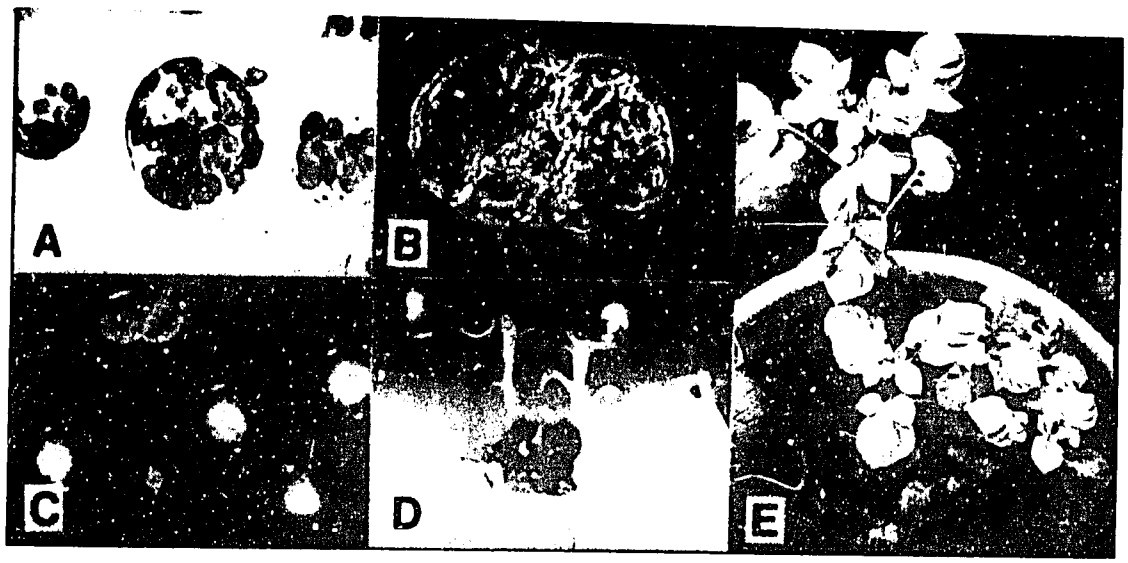


Figure 1. Regeneration of somatic hybrid plant between *Solanum tuberosum* and *Lycopersicon pimpinellifolium*.

A: A hybrid cell, B: Division of a hybrid cell, C: Calli from the fusion-treated protoplasts, D: Regeneration of shoots from a hybrid callus, E: A somatic hybrid plant.

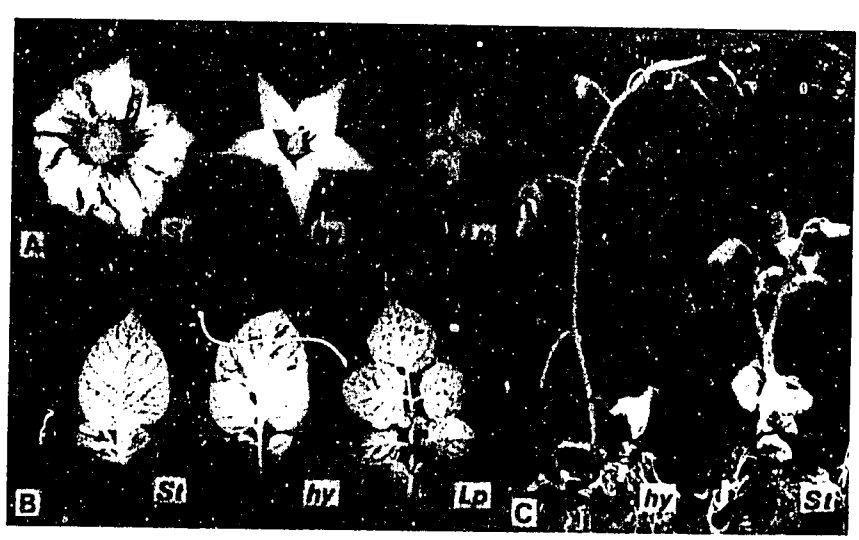


Figure 2. Flower (A) and leaf (B) morphology, and resistance to soft rot (C) of a somatic hybrid between *Solanum tuberosum* and *Lycopersicon pimpinellifolium*. St:*Solanum tuberosum*, hy:a somatic hybrid, Lp: *Lycopersicon pimpinellifolium*

Figure 3. Back-fusion and the produc.ion of asymmetric hybrid plant (BF13). Carrying the complete genome of potato plus a few chromosomes or chromosome fragments from *Lycopersicon pimpinellifolium*. PT31: disease resistant somatic hybrid between *Solanum tuberosum* and *Lycopersicon pimpinellifolium* St: *Solanum tuberosum* BF13: Asymmetric somatic hybrid produced by back-fusion of X-irradiated PT31 protoplasts with IOA-treated *Solanum tuberosum* protoplasts.

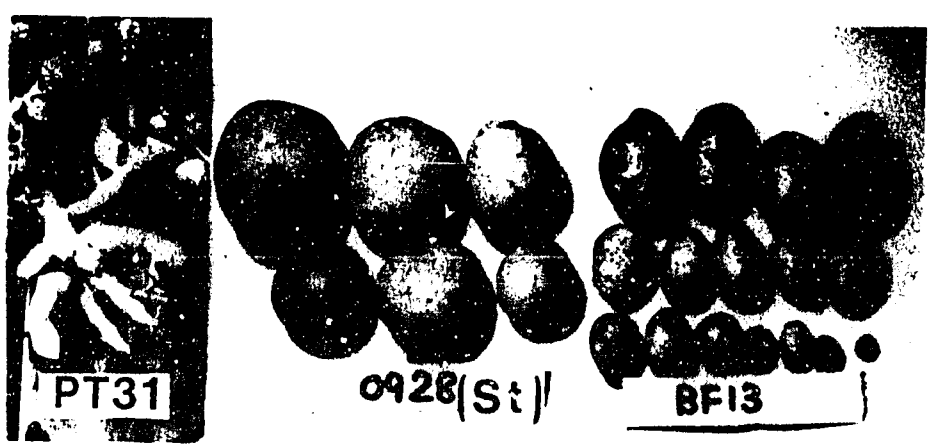


Figure 4. Tuber morphology of PT31, 0928 (*Solanum tuberosum*), and BF13 carries the complete genome of potato plus a few chromosomes or chromosome fragments from *Lycopersicom pimpinellifolium*. PT31: disease resistant somatic hybrid between *Solanum tuberosum* and *Lycopersicon pimpinellifolium* St: *Solanum tuberosun*. BF13: Asymmetric somatic hybrid produced by back-fusion of X-irradiated PT31 protoplasts with IOA-treated *Solanum tuberosum* protoplasts.

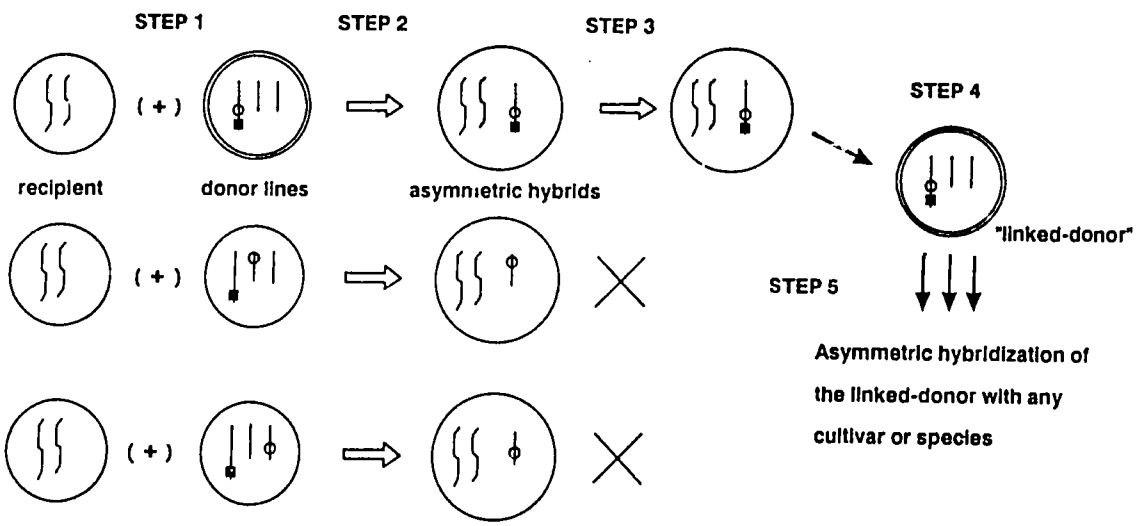


Figure 5. Nuclear (gene cluster) donor-recipient protoplast fusion. :an agronomically useful trait, O:a selectable marker

Step 1: Asymmetric hybridization of recipient with a set of donor lines with a selectable marker gene incorporated into different positions on the genome.

Step 2: Selection of asymmetric hybrids carrying the marker gene.

Step 3: Evaluation of the hybrids for the agronomically useful trait.

Step 4: Identification of the "linked-donor" lines. "Linked-donor" candidates can be selected also by linkage analysis.

Step 5: Reproducible transfer of the useful trait from the linkage-donors to any recipient through asymmetric hybridization.

The Application of Molecular Techniques for Potato and Sweet Potato Improvement in Latin America

L. Lago and S. Pérez[1]

The potato was an ancient cultivated plant before South America was colonized. We know that it was domesticated in this region and that it was dispersed by man over a considerable area by the Spaniards in the sixteenth century.

For many developing countries in Latin America and the Caribbean, agriculture represents both a short and a long term perspective of their economic development.

Potato production still averages less than 30 kg per person in developing countries, of which two-thirds is for human consumption. Human consumption in most developing countries is less than 20 kg per person per year, one fourth of the European average. Since this difference is due to the low yields obtained (Table 1), there is still considerable opportunity for increased potato production in the area.

Among the developing market economies, Latin America has the largest area planted in potato (1,066,000 ha). It is the second in production (11,222,000 t) and the fourth in yield (10.5 t/ha). By contrast the developed market economies produce in approximately the same area double the production with double the yield (t/ha).

Developing countries grow nearly all of the world's sweet potato. Nearly 80% are grown in Asia, just under 15% in Africa, and only 5% in the rest of the world.

In Latin America and in parts of the Caribbean, biotechnology is typically identified with plant tissue culture. The conventional understanding is, in a sense, a result of the scientific, technical and economic situation of our region.

Potatoes and sweet potatoes are the two species that have received the most benefits of plant tissue culture methods in the Region.

For potatoes and sweet potatoes, improvement at the molecular level in Latin America is taking place in Argentina, Brazil, Mexico, and Cuba and at the International Potato Center in Peru (CIP). In the Andean sub-region, this is the least utilized level. The foundation of the Center for Genetic Engineering and Biotechnology (CIGB) in Cuba contributes actively to the development of the molecular techniques in Central America and the Caribbean, and to a lesser

[1]Luis Lago, Jefe Dpto. Categorías y Grados Científicos; and S. Talavera, Licenciado, Center for Genetic Engineering and Biotechnology, La Habana, Cuba.

extent in the rest of Latin America. It is based on the high performance of the installation conceived to bring together all the possibilities that modern biotechnology, including genetic engineering, have introduced in agriculture.

While the governments of the region are, in general, aware of the importance of some biotechnologies they are not aware of the importance of molecular techniques.

The development and application of so-called biotechnology, including molecular techniques, will allow them to hasten the genetic improvement of the plant species. We consider that biotechnology has a unique strategic significance in that its development can contribute considerably to the quality of life in developing countries by providing solutions to plant survival problems and by increasing the nutritional value of some agricultural products.

The challenge to an international effort in the Region is how to stimulate the development and application of molecular techniques. This could be achieved through the formulation of a coherent program which rationalizes and optimizes the participation of CIP or other international organizations in the process of multinational cooperation in this field.

CIP should be perceived as an organization possessing adequate expertise to advise and help national programs or NARS (National Agriculture Research System) to identify areas of development, and to set up their own application of molecular techniques. At the same time, a national effort to develop this strategy is absolutely indispensable

The most crucial distinction between the *Green Revolution* and the *Bio-Revolution* is fundamentally the private character of the latter (Buttel, 1985). The task of gaining access to the molecular techniques in connection with the other techniques in potato and sweet potato improvement is the responsibility of the public domain and the private sector in the NARS. The prerequisite for the application of these techniques is the existence of scientifc and financial resources.

Significant advances in plant genetic engineering have occurred since 1985-86 and field demonstrations have recently been done or are in progress. Without any doubt, the momentum in this field is accelerating the application of DNA recombinant techniques to improve some crops such as potato and, in the near future, sweet potato, which will make a big contribution in the human diet in Latin America and the Caribbean.

State of knowledge on the application of molecular techniques on those crops

The potato is susceptible to many diseases, some of which are widespread. The principal disease agents are viruses. Virus infections are important limiting factors in potato crop production. Breeding programs for virus resistance have been carried out in some countries with partial success.

At the same time, there are several constraints to sweet potato production such as the marketing system, demand factors, and the production constraints themselves.

For Latin American countries, sweet potato production occurs where large percentage of the diet is protein deficient, therefore, any genetic improvement should aim at improving both the quality and yield of sweet potatoes in the region.

A promising solution for increasing demand may be to diversify sweet potato uses. In Cuba, sweet potato is also used for feeding livestock in special pork production programs.

Since 1984, genetic engineering research has been aimed at transfering gene coding for the resistance to herbicides, introducing the gene for the toxin protein of some strains of *Bacillus thuringiensis* which could protect the potato plant against some insects (lepidopter pests). We consider that in the next few years, the most spectacular impact of these techniques will be in engineering potato plant resistance to virus diseases such as PVX, PVY, PLRV; resistance to pests, or improvement of this tuber's nutritional value.

The use of plant transformation to introduce disease resistance is promising (Beachy, 1987; Hemenway, 1988). By this process, it will be possible to confer virus resistance to plants.

The breeding programs for improvement of sweet potato germplasm have certains limitations inherent to the crop. It has been suggested (Jaynes, 1986) that it is possible to utilize the *Agrobacterium* vector system to introduce genes encoding for a protein rich in essential amino acids in both potatoes and sweet potatoes.

The application of molecular techniques in sweet potato can be expanded to insert genes for disease and insect resistance, in addition to the synthetic protein gene, leading to the production of improved germplasm.

Present Status of Application of Molecular Techniques in Latin America

The main activities in molecular manipulation and genetic characterization carried out in the region are:

1. Identification and characterization of genetic markers Crop: potato.

Technique	Country
RFLP mapping (Restriction Fragment Length Polymorphism)	Uruguay CIP (Peru)

The methodology of RFLP is a promising tool for the selection of specific genotypes and the identification of disease-resistant genes in *Solanum* species. The International Potato Center is making the greatest effort in the region in this respect.

2. Molecular cloning of virus genes in bacterial plasmid or phage vectors. Crop: potato

Virus	Countries
PVX	Argentina, Chile
PVY	Argentina, Cuba, Chile
PLRV	Cuba, Argentina

3. DNA sequence technology.

Countries
Argentina
Cuba
Mexico

4. Methods for in vitro evaluation and quantification of virus replication. Target: protoplast culture. Crop: potato

Virus	Countries
PVV	Argentina,Uruguay,Chile
PVY	Argentina,Uruguay,Chile

5. Moleculai hybridization techniques for detection and identification of plant virus. Crop: Potato.

Virus	Countries
PVX	Argentina, Cuba, CIP
PVY	Argentina, Cuba, CIP
PLRV	Argentina, Cuba, CIP
PSTV	CIP

o. Plant transformation methodologies. Crop: potato.

Target	Countries
Improvement of plant virus resistance	Argentina, Cuba, Mexico, CIP
Improvement of pest resistances	Cuba
Improvement of nutritional value	CIP
Improvement of bacterial resistance	CIP

Crop: Sweet potato

Target	Country
Improvement of nutritional value	CIP- Cuba

It is clear that the biggest effort is directed to potato virus diseases (PVX, PVY, PLRV). We believe that more research work and technology transfer in the application the molecular techniques is needed for the sweet potato crop.

Other activities supporting molecular DNA technology are carried out in the region. Restriction enzyme production is achieved on the CIGB in Cuba. More than 30 different restriction and DNA modification enzymes are available from this Center.

We are now involved in the second conquest of the potato in Latin America by genetic engineering; not to introduce the potato in Europe, but to improve potato production.

References:

Beachy, R. N., P. Powell et al. 1987. Genetic engineering of plants for protection against virus diseases. CIBA Foundation Symposium pp. 151-169.

Buttel, F. H. 1985. From Green Revolution to Biorevolution. Some observation, on the Changing Technological Basis of Economic Transformation in the Third World. Econ. Develop and Cultural Change 34 (1).

Hemenway Cynthia et al. 1988. Analysis of mechanism of protection in transgenic plants expressing the potato virus X coat protein or its antisense RNA. The EMBO Journal 7(5): 1273-1280.

Horton, E. D. 1988. World Patterns and Trends in Sweet Potato Production and Use. Report of the First Sweet Potato Planning Conference. pp. 17-25.

Jaynes, J. M., M. S. Yang, N. O., Espinosa, and J. H. Dodds, 1986. Plant Protein Improvement by Genetic Engineering. Use of Synthetic Genes. Trends in Biotechnology 4:314-320.

Table 1. Potato production (Horton, 1985)

Region	Average 1980/82		
	Area (000 ha)	Production (000 t)	Yield (t/ha)
Developed Market Economies	2954	71210	24.1
Devoloping Market Economies	2918	32492	11.1

Table 2. World sweet potato production and yield. 1983-85 average (Horton, 1988).

	Production (10^6)	Yield (t/ha)	Prod. per capita (kg)
Asia CPE[a]	96	17	85
Far East	7	8	5
Africa	6	6	14
Latin America	2	7	6
Near East	1	6	3
All developing countries	112	14	32

[a] Centrally planned economies.

Application of Molecular Techniques for Potato Improvement in Africa

I. Robertson[1]

The only serious potato breeding that I am aware of in Africa is being done in Zimbabwe and South Africa.

Zimbabwe is currently producing foundation seed of the varieties Montclare, BPI, Pimpernel, Amythest, and Opal. Only two breeders were responsible for producing these varieties over the past 40 years. They replaced varieties such as Up-to-Date, which were very productive, but very susceptible to diseases, and which were chosen from variety trials in the 1930s selected from imported European varieties.

Since then, varieties such as Desire, Bintje, King Edward and Russet Burbank have been shown to do poorly in our climate, which is fairly hot. So far, those that CIP has kindly sent have not done any better.

We would like to incorporate features like bacterial wilt and tuber moth resistance from CIP's Kenya Station but so far that has not been possible. Anyway it would require the traditional crossing and subsequent back-crossing to incorporate it.

I offer this background because it may explain my desire to build the capacity of the soil of Africa to put useful genes into our own potato varieties with irrigation. We can currently achieve over 60t/ha in good seasons. We should like to preserve that high potential with added genes that confer resistance to pests and diseases and so enjoy high yields in the bad seasons--bad for insect invasions, bad for bacterial attack, bad for fungal infections, bad for weed explosions.

To report as requested, the state of the art in Africa is very easy, short but not sweet. I have heard of some useful potato tissue culture work in West Africa, in Morocco, and in Egypt. At a conference of Agrarian plant breeders and geneticists, the Egyptians said they had selected their own Bt gene and intended to put it in potato among other crops. I am in touch with friends in South Africa and they are using tissue culture on both potatoes and sweet potato, but so far there is no talk of gene transformation, though several of their labs have the capacity. It is not a priority crop.

In Zimbabwe we have brought each of our above-mentioned varieties into the lab, put them through a meristem cycle, and are in the process of multiplying them along the excellent CIP

[1]Ian Robertson, Crop Science Department, University of Zimbabwe, Harare, Zimbabwe.

guidelines. One commercial farmer has taken this up seriously, using University know-how. This is really to provide an alternative to the current traditional production system for the provision of foundation seed that is grown in our highland area, which is virtually aphid-free, and sold to growers in lower altitudes, who have to buy AA Seed every year.

The alternative (tissue culture and micropropagation) is needed in the interest of both competition and free enterprise to keep the price of seed down, and to provide a stand-by alternative in case the seed system breaks down, overcome by bacterial wilt.

However, three weeks ago, Andrew Connolly did transform our best variety Amythest, and the plantlets are growing on Kanamycin medium, and are testing as GUS-positive. Richard Jefferson, the patenter of the GUS gene, was kind enough to visit, courtesy of the FAO, and to teach our team and to leave with us some of his plasmids in their various bacteria. This is the sort of technology transfer that we very much appreciate. Our thanks to the FAO and to Dr. Lucas Brader.

The clones that we are sub-culturing from these transformants will be tested shortly, first in the greenhouse and then, if possible, in the field. We have now a regulatory advisory committee set up by our Zimbabwe Research Council that is deciding which of the various national guidelines and regulations will best suit our situation.

Looking toward the future as this planning conference does, our growers do have a wish list. The list is as follows, defined by growers. They would like potato leafroll resistance, which they have not successfully bred for. They would also like resistance to viruses X and Y. They would like resistance to cutworm, to lepidopteran, to potato leaf moth, and to leafminer fly.

However, the big need is for resistance to bacterial wilt: the seed growers observe very strict hygiene to keep it away; the regular growers, at lower altitudes, take losses year by year. Nematodes also take their toll each year; herbicide resistance would be quite a help.

There are really no figures to back these views as each season is different, however, the actual growers know what they want if they are convinced it is possible. Resistance to any one of these stresses would be a significant improvement, resistance to several would be regarded as almost a miracle.

Our total crop is not large--perhaps 1 million dollars a year from about 40,000 tons annually. Sadly, for several years we have turned down requests from AID agencies wanting to buy seed for our Mozambique neighbors, for farmers and for refugees, and as a result seed is shipped form Europe at vast expense and the varieties are not too appropriate.

If in Zimbabwe our lab could use the genes, and the plasmids, and the vectors that some of you are working so hard on, we could help alleviate some of our misery and begin to build to decent life for all our neighbors, including Angola, Zambia, and Namibia, where the capacity for this kind of work is zero.

Incidentally, potato is a crop that can stay underground until harvest so it is somewhat harder to carry off quickly on a hit-and-run bandit basis.

It is also a good crop with which to introduce the concept and practice of genetically-engineered varieties to both our legislator and to their officials.

Africa needs to become self-sufficient in food and then in earning-power to buy foreign goods and service. Modest use of already proven biotech could in theory double all our yields of every crop.

One reason why it is not easy to apply biotechnology is that the infrastructure, civil service, customs, licenses, inspection systems, forward planning for essential spares, fuel, and machinery gets bogged down in the network of mutual favors concerning jobs, decision-making, paper-movement, and so on in our emergent bureaucracies.

The imperative for this terrible paralysis is that no one is secure, no one can plan with confidence for next year, so everyone is milking the system while they can.

To alleviate this, could we not aim to provide some security by providing a few of the necessities of life virtually free? One solution is technical: to distribute high quality seed or planting material.

Let me remind of a simple graph (Fig. 1)

The wide dissemination of clean seed and planting material will close that gap on the graph. This is not impossible. On my return I will teach a training course to 8 field workers to see if we can offer them the simple scientific technique of microprogation plus elite starting clones. Our aim is to distribute this high quality material, delivering it to the most remote areas -- where it is most needed. We were inspired by stories from CIP of what the Vietnamese were doing.

A second way out is an act of lunacy, or so I have been told. Let me offer it to you for comment or commiseration.

I start from the fact that the US and the EEC each subsidize their food producers to the tune of 30 billion dollars a year. That means that the consumers, employed and unemployed, are getting some of their food virtually free -- at the taxpayers' expense.

Could we not turn this on its head and provide bread, salt, and potatoes almost free to all who care to come and collect them? Then, even the humblest person has a little security, a little confidence in the future, a little less reason to pester his relative who is underpaid, but who can hold up the paperwork until a favor is offered.

The reason that I have the temerity to raise this at CIP and with you is that the bottlenecks in Africa are not technical; they are at the interpersonal level.

I think, however, that those who handle and introduce this stunning new technology could use their leverage to help suggest more democratic solutions to these age-old problems.

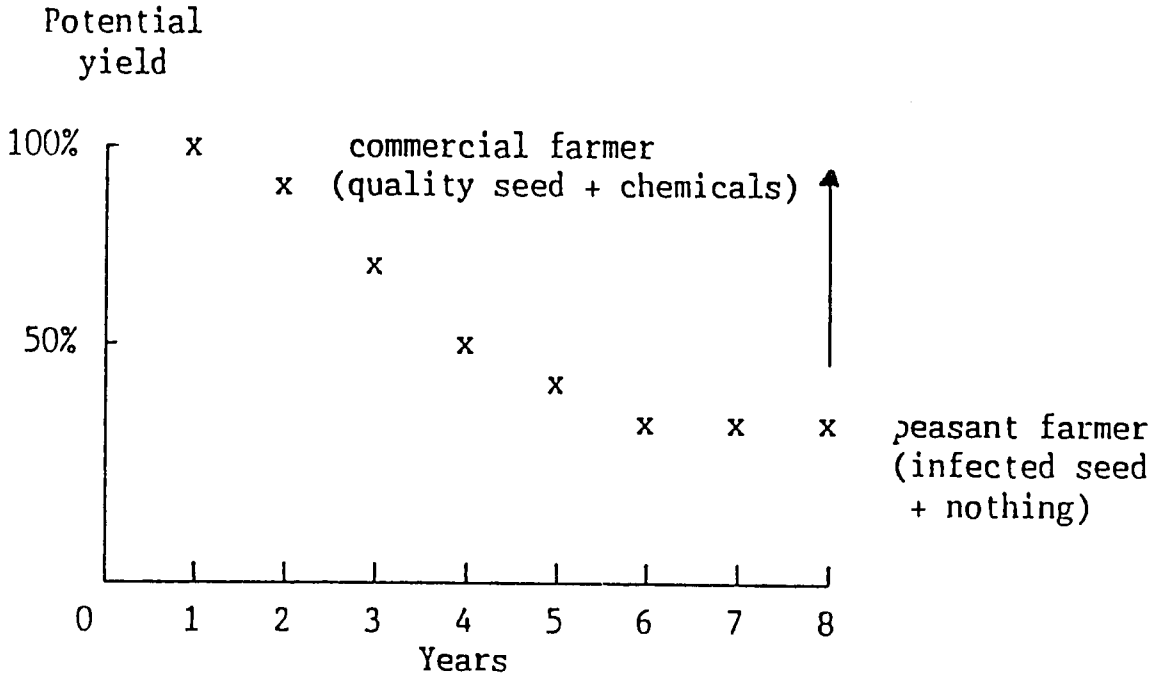


Figure 1. Yields achieved over a period of years starting from clean seed. The commercial farmer from the cash economy can stay near 100% by repeatedly buying new seed and using pesticides and herbicides as needed. The peasant farmer in Africa starts and finishes at the bottom of the graph as he always plants poor, retained seed. Closing the gap represented by the arrow is possible.

Application of Molecular Methods at CIP, Potato Improvement Through Gene Identification and Gene Transfer

J. H. Dodds[1]

In collaboration with the Biochemistry Department at Louisiana State University, CIP has developed a project to insert synthetic genes into potato plants by means of genetic engineering. In this project, *Agrobacterium* plasmid vectors (Jaynes et al., 1986; Dodds, 1987), are utilized in a system now used by research groups worldwide, including laboratories in Europe, the United States, Brazil, Mexico, and India. The primary objective of the project is to enhance the nutritional value of the potato by obtaining the supplementary production of a synthetic protein, rich in essential amino acids. The synthetic protein is produced by a gene that has been synthesized by machine in the LSU laboratory.

Through this research collaboration, the insertion of the synthetic gene into potato plants has been successful. Evidence has also been obtained that the gene is transcribed and produces a corresponding messenger RNA (mRNA) molecule, which is then translated in the plant to produce the synthetic protein (Espinoza et al., 1987). This synthetic gene project has shown that the technology now exists to genetically engineer genes into the potato. However, much more research, as well as the appropriate international legislative measures, will be needed before these genetically-engineered plants can be released to national potato programs.

On the basis of its success with the synthetic protein gene, CIP is directing attention to the possible use of genetic engineering to give the potato plant resistance to pests and diseases (Jaynes et al., 1987). Through collaborative links, CIP hopes to genetically engineer potato plants for resistance to viroid and virus infections by inserting gene sequences that will interfere with virus and viroid replication. This approach would allow potato plants to be grown for more generations in the field before degeneration of seed occurs.

Collaborative research is also being initiated in an attempt to genetically engineer resistance in the potato to diseases caused by bacteria such as *Pseudomonas solanacearum* and *Erwinia* spp. For these experiments, we are using genes that have been purified and that code for proteins with known, potent antibacterial activity. A genetic engineering approach to bacterial disease resistance may allow us to develop clones with resistance to both *Erwinia* spp. and *Pseudomonas solanacearum* in the same clone without major changes of the existing agronomically-acceptable characters of that clone.

[1]John H. Dodds, Head, Genetic Resources Department, International Potato Center (CIP), Lima, Peru.

Gene Identification

The characterization of genotypes has until recently been based on morphological analysis and the analysis of soluble proteins by the use of slab gel protein electrophoresis and isozyme analysis. What is in reality being analyzed are the products of genes as opposed to the genes themselves. Recent developments in molecular biology have allowed the development of a technique to restrict and probe plant genes. These techniques depend on the identification of polymorphisms in cut DNA fragments and are known as restriction fragment length polymorphism (RFLP) analyses.

RFLP analysis allows not only the study of taxonomic relationships between genotypes, but can also be used to map characters onto plant chromosomes. This mapping approach is already being applied in potato to locate specific resistance genes such as PVX and PVY resistance.

When distant crosses are made, i.e. cultivated x wild genotypes, RFLP analysis can not only indicate the amount of wild chromatin passed to the progeny, but may also be used as a screening tool to look for specific resistances.

In the case of potato, RFLP analysis using clones/probes from tomato has already allowed the mapping of approximately 140 loci, with loci marked on all 12 chromosomes (Tanksley et al., 1988). In the immediate future the needs are (a) a mammoth effort to map many more loci and (b) widening of probes/analyses to a greater diversity of *Solanums*, both cultivated and wild.

CIP's Research Network Approach to Biotechnology

The cost of initiating laboratories to carry out the more sophisticated aspects of biotechnology, i.e. gene synthesis/construction, RFLP analysis etc., is generally greater than can be afforded by a national potato program; an International Research Center must also balance biotechnological prospects against its other activities. At CIP we have taken an approach of collaborative networking for approaching the more sophisticated aspects of biotechnology. Many laboratories in developed and developing countries have expertise in specific aspects of biotechnology, and small amounts of funding can allow IARCs and/or National Programs to tap into this expertise. Figure 1 shows the way that CIP has built up a network of research activities based on comparative advantage. This involves collaboration on genetic engineering, and RFLP analysis as discussed in more detail in the next section.

This type of collaborative activity not only allows access to a wide range of expertise, but also allows project development in a highly cost-effective manner.

The Technology Transfer Process: Transfer of Technology or Fruits of Technology?

In the case of "sophisticated" biotechnologies, i.e. RFLP gene mapping or transformation studies, the technology transfer process should be considered. It may be that in many of these cases, the

technology per se would not be appropriate for transfer to a developing-country national program, however, the fruits of that technology, i.e. improved germplasm, may be distributed for evaluation by a National Program.

Especially in the area of transformation for production of new varieties, care is needed to ensure that programs do not become dependent on new varieties that may have import strings attached, e.g. herbicide resistance engineered for a specific herbicide that may be imported by a single agent, and the risk of major crop loss without access to the herbicide.

In this area the International Centers and Development agencies have an important role to play in defending the interests of National Programs.

Policy Implications

For Developing Countries

Developing-country National Programs have a wide range of biotechnological inputs from different sources. It is important that biotechnology not be seen as a panacea for all problems. It has much to offer, but it does not offer miracles. It is important that National Programs be able to evaluate the pros and cons of biotechnology. This will require varying amounts of manpower training/development. Although it is hard to generalize, we feel that most, if not all, National Programs should give attention to the following:

1. Evaluation of National Agricultural Problems and the existing technologies available to alleviate the problem.
2. Define priority areas where a biotechnological approach is seen to be a better alternative to conventional methods.
3. Avoid overlap with other crops, i.e. a multicrop approach.
4. Evaluate manpower and infrastructure available to carry out this type of work.
5. Maximize interaction with IARCs and other organizations backstopping biotechnology and try to network with neighbor countries in a region, thus reducing cost and duplication of effort.

For Development Agencies

It would be beneficial to give consideration to the following points:

1. Clearly define what constitutes research support, manpower development, and assistance to apply a proven technology.
2. Attempt to interact with the private sector, since an end product can be sold in cases of proven technologies (i.e. tissue culture-driven seed programs)

3. Distinguish science fiction from science fact in the area of biotechnology. Many National Programs need manpower development so as to appropriately evaluate the opportunities offered by a biotechnological approach.

4. View from a developing-country perspective the whole area of patents and intellectual property rights.

5. Work closely with the IARCs and other international scientific agencies to assist in research of scientific priorities and to ensure that the products of biotechnology reach the end users, especially the developing-country farmer.

References

Espinoza, N. O., S. F. Yang, J. M. Jaynes, and J. H. Dodds. 1987. Genetic engineering of the potato. Sci Am.(in press).

Jaynes, J. M., K. G. Xanthopoulos, L. Destefano, and J. H. Dodds. 1987. Increasing bacterial disease resistance in plants utilizing antibacterial genes from insects. Bioessays 6: 263-270.

Jaynes, J. M., M. S. Yang, N. Espinoza, and J. H. Dodds. 986. Plant protein improvement by genetic engineering: use of synthetic genes. Trends in Biotechnology. 4: 314-320.

Tanksley, S., M. W. Bonierbale, R. Plaisted. 1988. A genetic lingake map of potato. Theoretical and Applied Genetics. (in press).

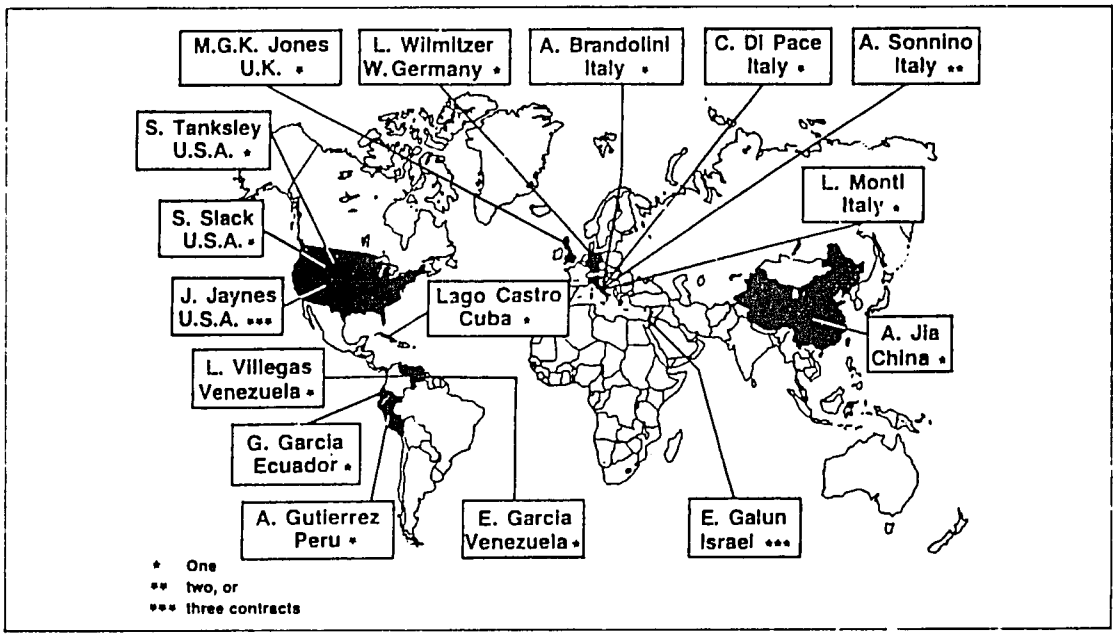


Figure 1. CIP collaborative biotechnology research agreements related to germplasm enhancement.

Use of Synthetic Genes to Enhance Nutritional Quality and Disease Resistance in Plants: Application to Potato

L. Destéfano-Beltrán[1], P. G. Nagpala[1], J. Kim[1], J. H. Dodds[2], and J. M. Jaynes[1]

Approximately ten years ago, we began thinking about the possibilities offered by the newer techniques of genetic engineering and biotechnology to modify plants in predictable and highly desirable ways. From our studies, we became firmly convinced that great potential existed for the application of these methods in at least two areas: to significantly alter the protein quality of plant foodstuffs and to enhance their overall disease-resistant properties. If successful, both of these areas of research could make a profound impact on increasing the amounts and quality of crops consumed by people and animals in the developing and developed countries of the world.

Improving the quality of plant protein

The biosynthesis of amino acids from simple precursors is a process vital to all forms of life as these are the building blocks of proteins. Organisms differ markedly with respect to their ability to synthesize amino acids. In fact, virtually all members of the animal kingdom are incapable of manufacturing some essential amino acids. It is generally agreed that humans require eight of the twenty common amino acids in their daily diets. Protein malnutrition can usually be ascribed to a diet which is deficient in one or more of these essential amino acids. A nutritionally adequate diet must include a minimum consumption of these compounds.

When diets are high in carbohydrates and low in protein over a long period, essential amino-acid deficiency results. The name given to this condition is known as "Kwashiorkor", which is an African word meaning "deposed child" (deposed from the mother's breast by a newborn sibling). This debilitating state of malnourishment, characterized by a bloated stomach and a reddish-orange discoloring of the hair, is more often found in children than in adults because of their greater need for essential amino acids during growth and development. In order for normal physical and mental maturation to occur, the daily requirements of amino acids must be met.

Essential amino acid content, or protein quality, is as important as total protein quantity or total calorie intake in the diet. The protein quality work began with a rat-feeding experiment using polylysine as a source of the essential amino acid lysine. It was found that this polymer could be utilized as an efficient source of free lysine in the rats' diet. The reported data showed the

[1]Luis Destéfano Beltrán, Pablito G. Nagpala, Jaeho Kim, and Jesse M. Jaynes, Department of Biochemistry, Louisiana State University, Louisiana, U.S.A.
[2]John H. Dodds, Head, Genetic Resources Department, International Potato Center (CIP) Lima, Peru.

fundamental role an adequate diet of essential amino acids, plays in normal animal development and overall physical well-being.

Some foods, such as milk, eggs, and meat, have very high levels of essential amino acids. On the other hand, most foodstuffs obtained from plants are poor sources of protein because of their relatively low content of some or all of the essential amino acids (Fig. 1). In general, the essential amino acids that are found to be most limited in plants are isoleucine, lysine, methionine, threonine, and tryptophan.

People inhabiting a typical developed country have few amino acid-deficiency problems primarily because their diet is composed of a mixture of a wide range of animal and plant proteins. That situation is, however, not true in many developing countries where up to 90% of total food intake, is from a single crop. Rice, for example, is the major staple in Asia while potato is the staple in the Andean region of South America. When there is heavy dependence on plant protein from a single source, its essential amino acid composition becomes of critical importance. In those situations, where the food source is a single plant, it would be highly beneficial to "engineer" the plant to produce proteins with a balanced essential amino acid content.

The innovative methods of genetic engineering offer a novel approach for modifying the essential amino acid composition of plant proteins, and can thus increase their nutritive value. The new approach is not to try to alter the existing plant proteins, but rather, to supplement existing plant proteins with new synthetic proteins with a high content of essential amino acids. To produce a synthetic protein of known amino-acid composition, a DNA fragment can be constructed with the appropriate codon sequence from a set of overlapping oligonucleotides. These DNA fragments can be produced using modern chemical techniques and can be ligated into various plasmid vectors. The cloning and expression of synthetic DNAs, containing repeated codons for a single amino acid, could be used as a means to supplement the essential amino acid-content of plant protein. However, as it was stated previously, plant proteins are deficient in more than a single essential amino acid, and therefore, the total amino acid composition of plant proteins must be taken into consideration.

Gene Design and Synthesis

Over the last decade, rapid advances have been made in the techniques of sequencing and synthesis of nucleic acids. It is now possible to synthesize DNA fragments with an individual length of up to 100 bases. Genes have been constructed and expressed in bacteria which code for proteins with a high content of the essential amino acids found to be the most deficient in plant-derived proteins. This was done in bacteria first in order to facilitate the analysis of the new genes and their new products (Jayness et. al., 1985). This work is important because it demonstrates that a gene could be synthesized *de novo* and the encoded protein could be efficiently expressed in a bacterial system.

This "first generation" gene fragment (the gene has been named HEAAE I for High Essential Amino Acid Encoding) is 292 bp long and the proteins encoded represent polypeptide sequences derived from both possible reading directions of the HEAAE-DNA. That is, HEAAE-DNA is constructed symmetrically so that a protein, containing a high content of essential amino acids, will be produced no matter which strand of the synthetic DNA is read by a host's cell protein-synthesis machinery. The compositions of A and B proteins considered the most deficient in essential amino acids of plant-derived proteins, (Hansen, 1979) are three-fold higher than those found in milk or egg protein.

Next, the HEAAE-DNA fragment has been inserted into the coding sequence of the chloramphenicol acetyl transferase (CAT) gene to encode for a CAT-HEAAE fusion protein. The chimeric gene is under the control of the nopaline synthetase promoter and the 3' end of the nopaline synthetase gene for polyadenylation. The construct was introduced into the agropine-producing *A. rhizogenes* strain R1000 by direct transfer and double-recombinants containing the construct were selected on appropriate antibiotic markers. In vitro-derived potato plantlets were inoculated with the transconjugant *A. rhizogenes* and hairy roots were obtained after two weeks. Regeneration of these hairy roots yielded transgenic potato plants expressing the chimeric protein. Southern and Northern analysis showed the proper integration into the genome and expression of the chimeric mRNA. Western blots confirmed the presence of a chimeric protein of the expected size, 38 Kd (Fig. 2). The CAT protein is known to be approximately 25 Kd in mass and the HEAAE-protein, based on computer analysis, is about 13 Kd. (plant Science, 1989. 64.99-11). This first attempt did not result in any significant increase in the levels of essential amino acids, as indicated by amino acid analysis of total tuber-protein. This was due to a low level of expression, instability of the protein and the fact that the HEAAE-protein was "diluted" in the CAT-HEAAE fusion protein.

Design of an improved synthetic protein: HEAAE II

While detectable levels of HEAAE I were obtained in potato plants, a new synthetic protein soon became necessary. A decision to design a new protein in a systematic and logical way was made. First, from the amounts of essential amino acids necessary for normal metabolism of people of all ages, a set of numbers were deduced: the "needs ratio." Also, "deficiency values" or the ratios of deficient essential amino acids for the primary crops people consume throughout the world were determined. From all these data, the ratios of essential amino acids needed to totally complement each particular plant foodstuff were derived. The averaging of these values yielded a set of numbers known as the "average ratio for all crops idealized to the HEAAE II monomer." This set of numbers represents the ratio of essential amino acids necessary to complement the deficiencies found in all 12 crops for all human age groups.

From the above set of numbers, a HEAAE II protein was designed (Fig. 3). It was constructed in such a way as to be highly stable with a very high degree of aggregation reminiscent of plant-storage proteins. In fact, a monomer of this protein (synthesized in a peptide machine)

has behaved as a hexamer in gel-filtration studies. Also, circular dichroism analysis of the monomer has indicated that the protein is almost 100% alfa-helical in salt concentrations of up to 6M (J. Jaynes et al, unpublished results). The gene was assembled in a step-wise manner until a gene encoding a tetramer of the protein was obtained (Fig. 4). The construct has been introduced into tobacco plants via *A. tumefaciens*-mediated transformation. Preliminary data seem to indicate that leaves of transgenic plants accumulate much more "total" protein, ~ 14% in control plants vs. almost 50% (dry weight) in some of the transgenic data. This unexpected effect is still unclear and experiments are underway to determine the cause of such an increase in the level of total protein.

This method of gene design is flexible enough to produce proteins possessing any particular amino acid composition. Therefore, proteins could be specifically designed to supplement any desired animal feed or human food. It should be pointed out that the insertion of lysine at frequent intervals in these synthetic proteins provides numerous sites for proteolytic attack by tripsin (a digestive enzyme). This feature is important as it increases the bioavailability of the supplemental protein. Currently, experiments to introduce this gene into potato are underway and soon it will be possible to assess its effect in the total tuber-protein levels of transgenic plants.

Enhancing disease resistance in plants

The welfare of most of humanity is inextricably bound to the efficient cultivation of plants. Plant disease is a disruptive and, at times, catastrophic occurrence. For instance, late blight, a fungus disease, caused the starvation of one million people and forced the immigration of another two million to North America due to its decimation of the potato crop (the disastrous Irish Potato Famine of 1845-1860). It is estimated that as much as one third of the total crop losses in the world can be directly attributed to plant disease (Fig. 5). The loss in the potato, associated with bacterial disease, can be as high as twenty-five percent (about US$4 billion annually) of the total worldwide production.

Plant breeding, the movement of desirable traits between plants using the traditional Mendelian techniques, has played a major role in modern agriculture by providing plants with increased disease resistance and higher yields. However, there are inherent problems with this method. For example, it may take as long as fifteen years to introduce, select, and establish a particular trait into a commercial cultivar and, very often, some traits of agronomic importance are impossible to introduce using these conventional techniques.

When confronted with a disease problem, the plant breeder has relied on the fact that somewhere, among plants of the same or closely related species, an individual can be found which retains resistance to that particular disease pathogen. This single plant can then be incorporated into the breeding program by introducing its genes into already well-accepted, traditional varieties, through the use of sexual crosses. It is assumed that this will eventually produce a hybrid plant which retains all of the desirable traits including the new one, resistance.

Disease pathogens, like all other organisms are constantly forged on the "anvil" of natural selection and are very efficient in eventually overcoming a plant's defenses. So, a continual war of attrition has developed between the pathogen and the plant breeder, with no apparent winner so far. One of the main weaknesses of traditional plant breeding is its dependency upon sexual crosses which are limited to the genes that exist in only one relatively small group of organisms; the species. Recombinant DNA, the manipulation of genes in the test tube, allows the transcendence of inter-species barriers and makes novel genetic combinations possible.

Genetic engineering promises to accelerate the breeder's efforts and although, as yet, there are no transgenic cultivars available in the marketplace, these new techniques have yielded important breakthroughs in obtaining plants with an increased resistance to virus, herbicides, and insects. Insect-tolerant plants are an example of how genetic engineering has increased the gene pool available to the breeder. Traditionally, breeders relied on the existence of alternative characteristics found among plants of the same or closely related species. By using the insect-toxin gene of *Bacilus thuringiensis* (B.t.), researchers have opened new doors for crop improvement.

In contrast to insect-tolerant plants engineered with a bacterial insect-toxin, microbial-tolerant plants have been obtained by using insect-derived lytic peptides. In this section, we will describe our initial experiments with lytic peptides and how we are utilizing their genes to endow plants with new weapons to fight bacteria and fungi.

Bacterial and fungal diseases of potato

The Peruvian potato (*Solanum tuberosum* L.) is susceptible to a great number of diseases, some of which are of worldwide importance whereas others are only significant locally. The casual agents include bacteria, fungi, viruses, viroids, mycoplasms, and nematodes. There are more numerous diseases in potato grown in the tropics than those cultivated in temperate areas. Indeed, it has been reported that "*Solanum andigenum-tuberosum* is afflicted with at least 175 different diseases in the tropics compared to 91 found in the temperate areas." Of those in the United States, ten have been reported to be caused by bacteria and thirty from fungi. Total crop losses in the potato resulting from bacteria and fungus diseases can amount to hundreds of millions of dollars (US$) annually in developing countries. During particularly bad years, damages can approach several billion dollars worldwide. The extent of loss varies greatly form country to country and is influenced by climate and conditions of growth and storage.

In the last 15 years, there has been a tremendous effort expended to introduce new varieties appropriate for the tropical areas. One primary component in restricting potato production in those regions is the presence of bacteria and fungus diseases. Among the most important are bacterial soft rot, caused by *Erwinia carotovora* and bacterial wilt (Brown Rot), caused by *Pseudomonas solanaceaurum*. Total losses from these two diseases can be from 30-100% between cultivation and the 2-6 month storage period, where temperatures can reach 27-32°C. The most important fungus diseases are caused by *Rhizoctonia solani, Alternaria solani, Verticillium dahliae, and Phytophthora infestans.*

Humoral immunity in insects

Humoral immunity can be induced in insects by an injection of either live, nonpathogenic bacteria or heat-killed pathogens. this phenomenon has been studied by many investigators in a number of different insects, especially those of the orders *Lepidoptera* and *Diptera*. However, diapausing pupae of the giant silk moth *Hyalophora cecropia*, has proven to be particularly useful (mainly because of their large size) to study the humoral defense mechanisms of insects. When *H. cecropia* pupae are immunized they produce a set of ~ 15 proteins which are normally not present in the hemolymph of the animals. Boman and his co-workers have exploited this fact, both in the purification of these immune proteins and in the isolation of mRNA, which was used for the preparation of a cDNA bank. After a short period of RNA synthesis, the insects respond to the exposure of bacteria by producing a potent antibacterial activity which is due to the synthesis of three (of which two are novel) classes of bactericidal proteins: cecropins, attacins, and lysozyme. The three principal cecropins, A, B, and D, are small basic proteins with a comparatively long hydrophobic region. The attacins are larger and there are two main forms: basic and acidic. The lysozyme protein is very similar to that found in chicken egg white (Boman and Hultmark, 1987, and references therein).

Cecropins

The cecropins are the most potent group of the antibacterial agents found in the *cecropia* hemolymph. These peptides possess a broad spectrum of antibacterial activity against both gram negative and gram-positive forms. They are small, 35-37 amino acid residues, strongly basic, and comprise three major forms: A, B, and D. Comparison of the amino acid sequence of the different forms has revealed a high degree of homology, ~60-80%. The peptides all have a basic N-terminal region and a hydrophobic stretch in the C-terminal part of the molecule. It seems that the cecropins are products of three related genes which, as in the case of the attacin genes, originated by gene duplication. Recombinant cDNA clones, corresponding to the cecropin B form, have been isolated. When analyzed together they showed that cecropin B is processed from a 62-amino acid precursor chain including a 26-amino acid leader peptide and a C-terminal Gly residue which is decarboxylated to render an amidated Leu. Recently, several genomic clones have been characterized and cecropin B was shown to exist in at least 3-5 copies in the *cecropia* genome.

Additional cecropin-like lytic peptides have been described in *Antheraea pernyi*, *Bombyx mori*, and *Drosophila melanogaster*. The sarcotoxins: IA, IB, and IC, were isolated from the hemolymph of *Sarcophaga peregrina* larvae (Boman and Hultmark, 1987 and references therein). These peptides, although small and basic, possess a somewhat different structural motif form that exhibited by the cecropins. However, all appear to act by causing generalized membrane disruption.

Attacins

Attacins are the largest antibacterial molecules found in the hemolymph of immunized *H. cecropia* pupae, with a molecular weight of about 20,000 daltons (20 Kd). They are comprosed of six different forms, which can be fractionated according to their isoelectric point. The results from the amino acid sequence of the N-terminus of five of the attacins indicate the presence of three basic and two acidic forms, which are slightly different from each other. It has been suggested that they are the products of two related genes. Two cDNA clones corresponding to these two genes, pCP517 and pCP521, were isolated. Comparison of the two clones has revealed 76% homology in the coding region and it has become evident that the genes originated from a common ancestral gene. The six attacin forms found in the hemolymph are probably products of secondary modification of the two precursor molecules (Boman and Hultmark, 1987, and references therein).

Lysozyme

H. cecropia lysozyme was purified from immune hemolymph and was identified as a chicken-type lysozyme. The primary structure of this enzyme was elucidated by determination of the complete amino acid sequence. It is composed of 120 amino acids with considerable similarity to vertebrate lysozymes. The amino acid regions responsible for the catalytic activity and for the binding of the substrate are conserved (Engstrom et. al., 1985).

Preliminary Experiments

Initially, it was reported that the cecropins possessed a strong bactericidal activity against several bacteria, including some animal pathogens. Because of the peptide's broad activity, it seemed reasonable to test its effect on plant pathogenic bacteria. Consequently, we synthesized cecropin B on a Biosearch Sam II peptide synthesizer and determined that it possessed substantial lytic activity against some of the most important plant pathogenic bacteria. Also, phytopathogenic fungi were tested and found to be sensitive to the peptide. These results have no antecedents in the literature. In fact, it was stated that the activity of cecropins was limited to bacteria with no effect found against any eukaryote (Boman and Hultmark, 1987, and references therein). These preliminary data were so encouraging that they provided us with the impetus to proceed further with the cloning and integration of the gene for cecropin B into plants. In order to obtain proper expression in the plant, it was necessary to introduce some modifications to the native sequence of the peptide. Therefore, we synthesized SB-37 and tested it against the same phytopathogens, with similar results (Fig. 6).

SB-37 differs form cecropin B in that two extra amino acids are added to the N-terminus of cecropin B (Met followed by Pro), Met-11 substituted by a Val, and a C-terminal Gly. These N-terminal alterations do not destroy the bactericidal activity. This would seem contradictory to results obtained in other laboratories (Andreu et al., 1987). In addition, the presence of a C-terminal Gly, instead of the amidated Leu, has no affect on the lytic activity. Furthermore,

significant changes in the amino acid sequence of cecropin B actually enhance biological activity as described recently by J. Jaynes and colleagues.

The "*de novo*" peptide, Shiva I, designed to have significant differences in sequence homology, while conserving the overall charge distribution, amphipathy, and hydrophobic properties of the natural cecropin B, has opened previously unexplored possibilities as new peptides in the Shiva series are being tested. Other classes of lytic peptides have been recently designed and tested with exciting results (manuscripts in preparation). In fact, the different types of peptides produced so far have proven to be more effective than cecropin B and SB-37 against several kinds of bacteria (Fig. 7).

An important characteristic of the humoral immunity of cecropia is that all these antibacterial proteins seem to act on different targets of the bacteria cells (Boman and Hultmark, 1987, and references therein). This coordinated attack surely results in an overall enhanced activity as compared to that of the components alone. Therefore, it seemed important to test as much of the total system as possible. Due to the lack of *cecropia* lyzozyme and the attacins, we settled for the chicken egg-white lysozyme, which is commercially available. The results are quite exciting as both peptides exhibited a pronounced synergistic effect in the presence of sublethal concentrations of the hen lysozyme. This supports the notion that combining several antimicrobial genes into one cultivar will render the plant more resistant to pathogen attack.

Introduction of the "Disease Resistance" Genes into Tobacco and Potato Plants

Tobacco was selected as a model system to test the efficacy of our constructs in enhancing plant disease resistance. Also, established tobacco transformation and regeneration protocols were available. Most of our constructs have already been introduced into tobacco and F1 progeny are currently being evaluated for increased disease resistance. Prior to selfing, all mother plants were screened for kanamycin resistance and GUS activity as appropriate and Southern analysis demonstrated the integration of single-copy genes. Northern and western analyses of some of the lines have shown the proper expression of the respective genes.

We have adopted a two-pronged approach to incorporate these genes into potato. Because of our previous experience with *A. rhizogenes*, we decided to start with our binary constructs, i.e. pBI 121-derivatives, in *A.rhizogenes* strain R1000 and infected stems of *in vitro*-grown potato plants (Desiree, LT-9 and an unnamed new cultivar from the Philippines, tentatively designated 86007). As expected, GUS-positive and kanamycin-resistant hairy roots were obtained after two weeks of infection and plantlets were regenerated 3-4 weeks later. These plants have been evaluated and Southern analysis has confirmed the stable incorporation of these genes. The other approach utilized *A. tumefaciens* strain LBA 4404 containing the pBI 121-derivative constructs to infect leaf-disks of *in-vitro*-grown Desiree plants. GUS-positive and kanamycin-resistant plants have been obtained after a few weeks. At present, most of the transformed lines are being

propagated in order to conduct the pathogen-challenge test with *P. solanacearum* and *E. carotovora*.

Bacterial Challenge

A. tumefaciens strain LBA 4404, containing the binary vector pWIShiva-1 (wound inducible), was used to infect tobacco leaf disks utilizing established protocols. Eventually, a number of transgenic plants were obtained and shown to be kanamycin-resistant and GUS positive. Southern blots have confirmed the presence of single copies of the Shiva-1 gene integrated into the genomes of these plants. Northern analysis has verified that the expression of the gene is triggered by mechanical and pathogen-induced wounding. F1 plants demonstrate a 3:1 segregation pattern for kanamycin resistance and in preliminary disease-challenge experiments, these plants exhibit delayed symptoms, reduced disease severity, and lower mortality after infection by *P. solanacearum* when compared to transgenic control plants (Fig. 8).

Tobacco plants containing the SB-37 gene under the 35S promotor have shown to be as sensitive as the control plants when challenged with *P. solanacearum*. In some cases they seem to develop symptoms and die earlier. Unpublished observations have shown in many systems a proliferative effect of the peptides at very low concentrations. These plants may have provoked a proliferative rather than a lytic effect on the pathogen (Jaynes et al, manuscript in preparation).

Our in vitro data consistenly showed *P. solanacearum* to be the bacterial plant pathogen with the highest LC50 for all our peptides. Most other pathogens are more sensitive, with values in some cases in the submicromolar range (Fig. 6). These results are encouraging, since introduction of the genes for SB-37, Shiva-1, and other lytic peptides into plants, where these other types of bacteria cause significant crop loss, may result in an enhanced resistance similar to that found for the tobacco plants expressing Shiva-1.

In 1987, we first proposed that lytic peptide-encoding genes could be used to enhance overall resistance against bacteria and fungus diseases. Recently, it has been suggested that the transfer and expression of a new class of peptides found in honeybee, the apidaecins, could generate similar effects in plants (Carteels et. al., 1989). However, since these peptides lack lytic activity and are merely bacteriostatic, the significance of this difference still must be determined.

Even though the lytic peptides are the most active components at hand, we must not disregard the potential of the other antibacterial proteins in our overall strategy. The giant silk moth's humoral immune system has been molded for millions of years through the evolutionary process and it demonstrates the wisdom of having a multiple-defense system in which the different elements can work in synergy with one another. This multi-level defense system would present a formidable challenge to the invading microbe, one which would be very difficult to evolve a means to circumvent. The probability that a pathogen would become naturally resistant to all three components at once is rather remote.

The promising results so far are merely the beginning. The extreme malleability of peptide design portends many new avenues of research, not only in the arena of plant-disease control, but also in human and veterinary medicine.

Any scheme of gene insertion into plants, for enhanced disease resistance, must consider the use of our new peptides. Not only are they extremely active against gram negative and gram positive bacteria, but substantial fungicidal properties are exhibited. Surprisingly, we have just discovered that several of the peptides kill plant nematodes. The consequences of this latest finding must be evaluated, but it demonstrates the versatility of these biomolecules. Someday soon, a single gene may eventually confer resistance in plants to most bacteria, fungus, and nematode diseases.

Conclusion

Genetic engineering offers the possibility of introducing a single trait, without altering other agronomically-important characters, into a well accepted or traditional plant variety, thus "fine-tuning" specific cultivars. This technology will allow the incorporation of desirable and inheritable traits into plants, with some ease, rapidity, and with a high probability of success when allied with modern plant tissue culture techniques. The use of these new methods, however, should be coupled with conventional breeding in order to achieve plant modification. When used together, the scope is greatly enlarged for the possibility of plant improvement.

The world is a vast repository of potentially valuable genetic material locked within organisms by the natural barriers which isolate species. Recombinant DNA technology allows the Earth's genetic resources to be exploited by providing a means of overcoming these barriers. Indeed, the potential for a new horizon in plant breeding is here and it may be possible, sometime in the near future, to endow plants with improved protein quality and permanent resistance to a variety of pathogens using existing genes or designing totally new ones.

We face a great challenge over the next few decades in providing enough high quality food to humanity. Application of the new and exciting techniques of biotechnology will help to ensure this birthright for all peoples of the world.

References

Andreu, D., R. B. Merrifield, H. Steiner, and H. G. Boman. 1987. N-Terminal analogues of Cecropin A: Synthesis, Antibacterial Activity and Conformational Properties. Biochemistry 24:1683-1688

Boman, H. G. and D. Hulmark. 1987. Cell-free immunity in insects. Annu. Rew Microbiol. 41:103-126

Casteels, P., C. Ampe, F. Jacobs, M. Vaeck, and P. Termps. 1989. Apidaecins: antibacterial peptides from honeybees. EMBO J. 8:2387-2391.

Engstrom, A., K. Xanthopoulos, H. G. Boman, and H. Bennich. 1985. Amino Acid and cDNA sequences of Lysozyme from *Hyalophora cecropia*. EMBO J. 4:2119-2122.

Hamsen, R. 1979. Food intake index. In Nutritional quality index of food. AVI. pp 169-543.

Jaynes, J. M., P. Langridge, K. Anderson, C. Bond, and C. W. Newman. 1985. Construction and expression of synthetic DNA fragments coding for polypeptides with elevated levels of essential aminoacids. Appl. Microbiol. Biotech 21:200-205.

Jaynes, J. M., K. Xanthopoulos, L. Destefano Beltran, and J. H. Dodds. 1987. Increasing bacterial disease resistance in plants utilizing antibacterial genes from insects. BioEssays 6:263-270.

Newman, C. W., J. M. Jaynes and D. C. Stands. 1980. Poly-L-lysine: a nutritional resource of lysine. Nutr. Rep. Int.: 707-715.

Sawyer, R. L. 1984. Potatoes for the developing world. International Potato Center. Lima, Peru.

Vaeck, M., A. Reynaerts, H. Höfte, S. Jansens, M. De Beuckeleer, C. Dean, M. Zabeau, M. Van Montagu, and J. Leemans. 1987. Transgenic plants protected from insect attack. Nature 328, 33-37.

Van Hofsten, P., I. Faye, K. Kochum, J. Y. Lee, K. Xanthopoulos, I. A. Boman, H. G. Boman, A. Engstrom, D. Andreu, and R. B. Merrifield. 1985. Molecular cloning, cDNA sequencing, and chemical synthesis or cecropin B from *Hyalophora cecropia*. Proc. Natl. Acad. Sci. USA 822240-2243.

Wellman, F. L. 1972. Tropical American Plant Disease. Scarecrow, Metuchen, N. J.

Xanthopoulos, K., J. Y. Lee, R. Gan, K. Kochum, I. Faye, and H. G. Boman. 1988. The structure of the gene for cecropin B, an antibacterial immune protein from *Hyalophora cecropia*. Eur. J. Biochem 172:371-376.

Yang, M. S., N. O. Espinoza, P. G. Nagpala, J. H. Dodds, F. F. White, K. L. Schnorr, and J. M. Jaynes. 1989. Expression of a synthetic gene for improved protein quality in transformed potato plants. Plant Sci. 64:99-111.

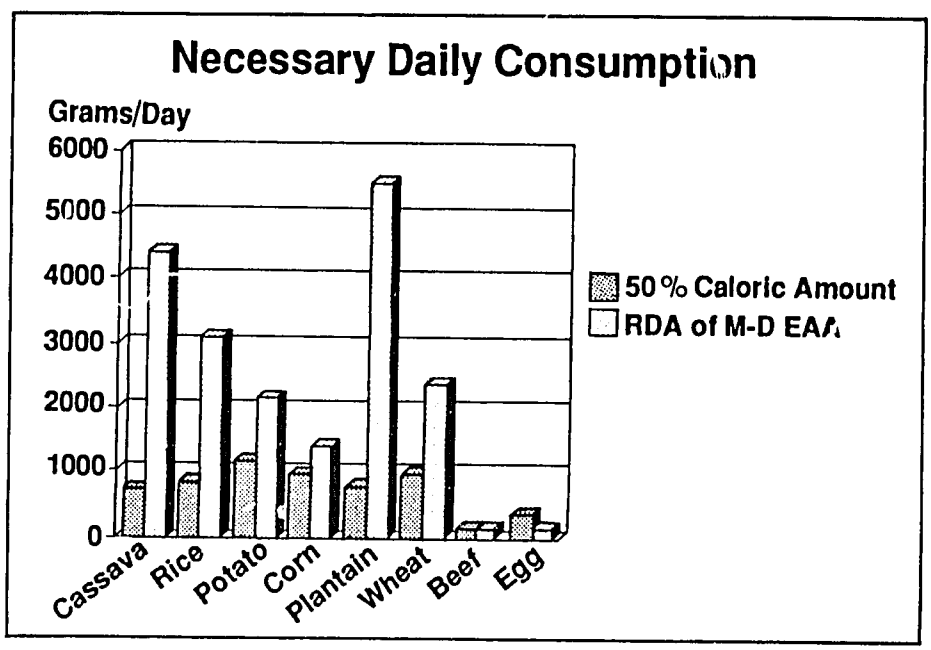


Figure 1. Necessary daily consumption of some of the most important animal and plant protein sources in grams per day to meet 50% of the daily caloric intake (▒) and the required daily amount for the five most-deficient essential amino acids (▒).

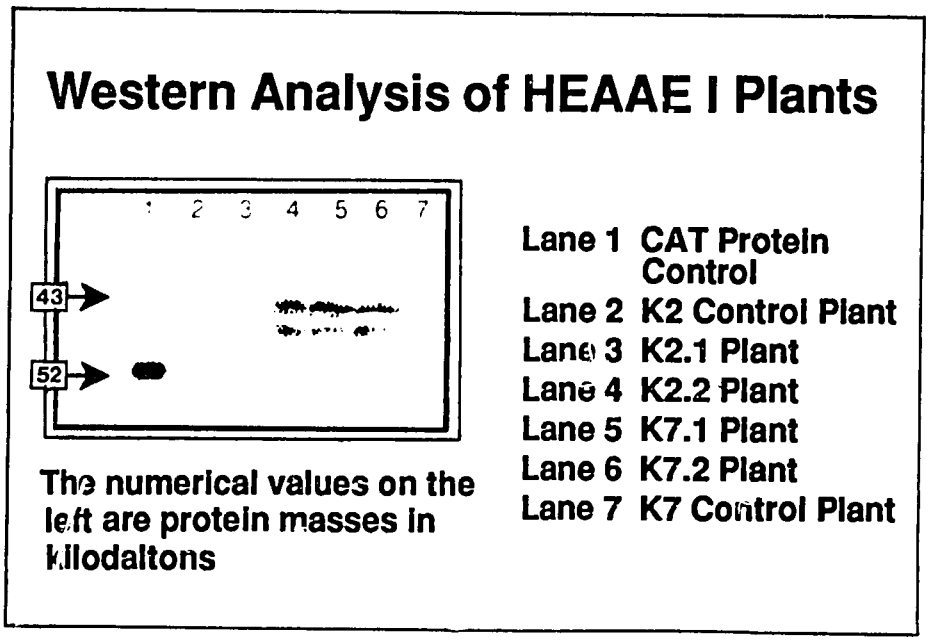


Figure 2. Western blot of total tuber protein. The chimeric CAT-HEAAE protein was detected with an antibody raised in rabbits against purified CAT. Level of expression ranges from 0.02 to 0.35% of the total tuber protein (K2.1 and K7.2 plants respectively).

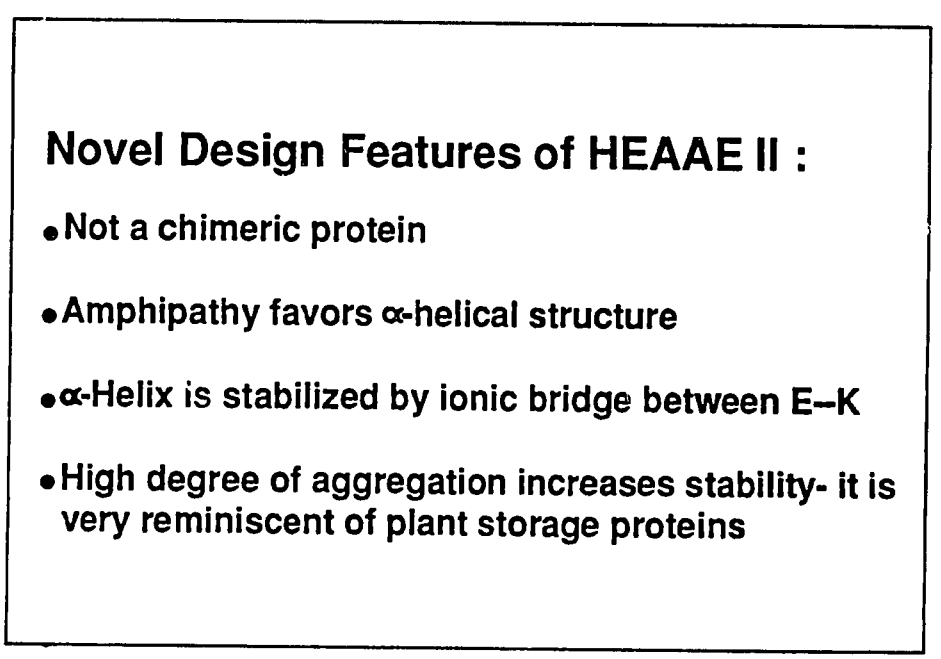


Figure 3.

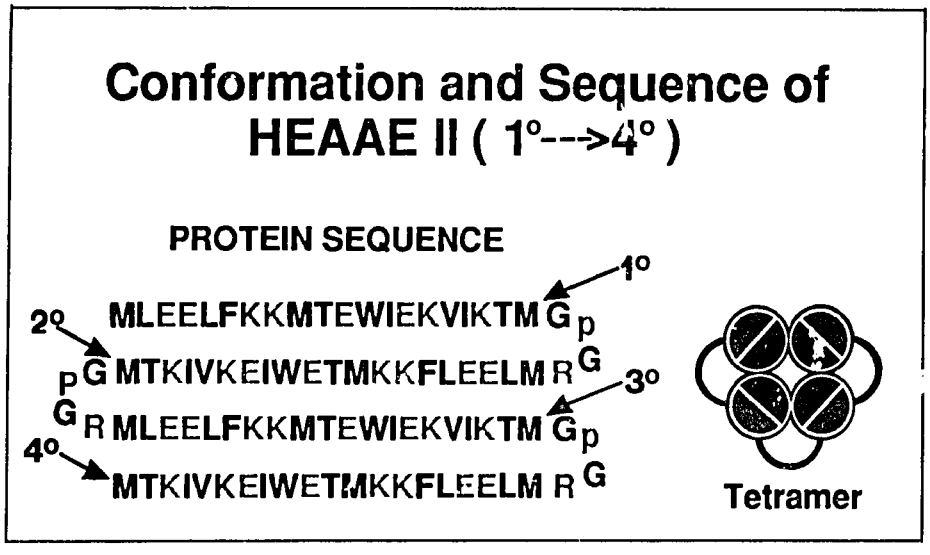


Figure 4. Sequence of the HEAAE II tetramer and its possible (idealized) conformation in solution. In black, hydrophobic and internal sides of the helices and, in gray, hydrophilic and external sides respectively.

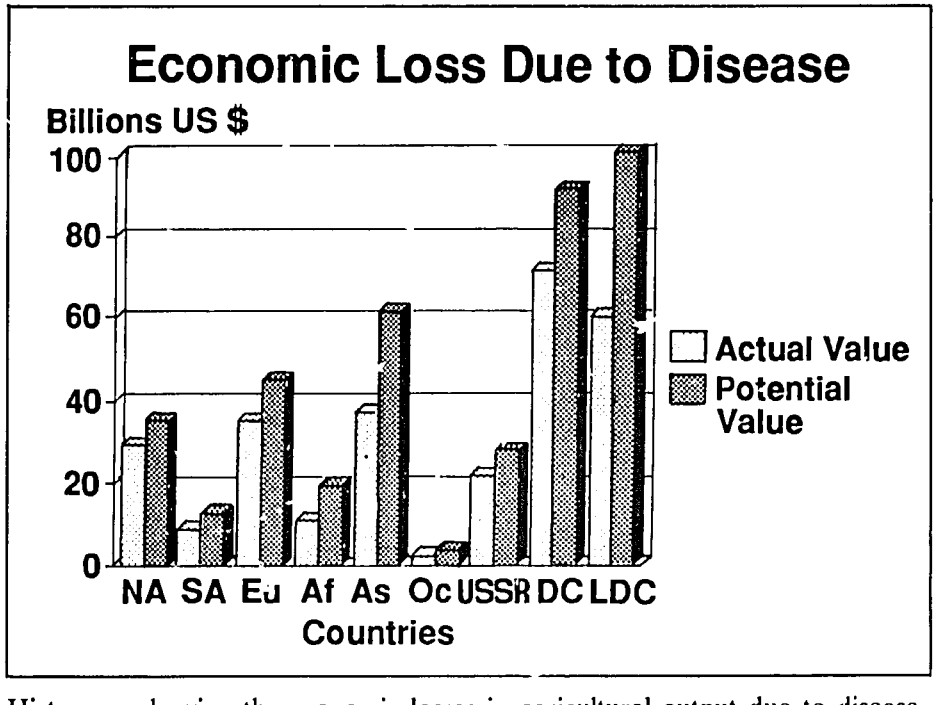


Figure 5. Histogram showing the economic losses in agricultural output due to disease. Losses are more significant in less developed countries (LDC). NA, North America; SA, South America; Eu, Europe; Af, Africa; As, Asia; Oc, Oceanic; USSR; DC, developed countries

Concentration in µM to Kill 50% of Test Bacteria

Bacteria	SB-37	SB-37*	Synergy	Shiva-1	Shiva-1*	Synergy
Pseu. syr.	5.2	0.19	27x	1.5	0.11	14x
Pseu. sol.	64.0	16.0	4x	40.0	7.0	6x
Erw. caro.	1.5	0.44	3x	0.5	0.26	2x
Xanth. cam.	0.57	0.03	21x	0.36	0.07	5x
Clav. mich.	2.7	0.15	18x	10.0	5.3	2x

*denotes the use of lysozyme; #'s of test bacteria between 50 and 100 million

Figure 6. Antibactericidal activity of SB-37 and Shiva-1 Synergy assays were performed under sublethal concentrations of both peptides and lysozyme. In general, a determined amount of bacteria was incubated with different dilutions of peptides for one hour, serial ten-fold dilutions of these were plated and the percentage of bateria killed were determined from control incubations with buffer.

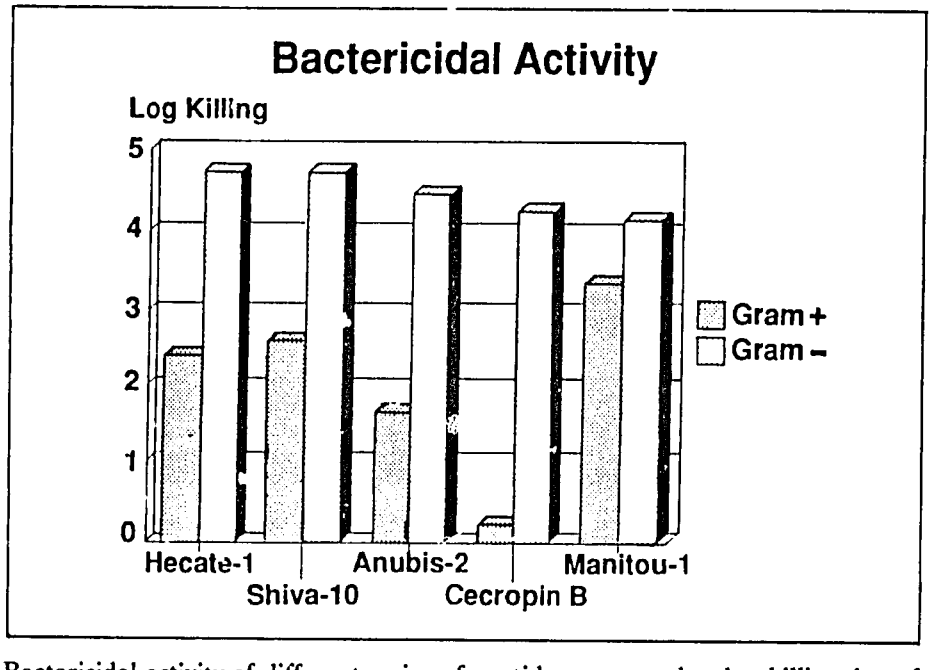


Figure 7. Bactericidal activity of different series of peptides expressed as log-killing: log of the ratio of initial number of bateria to the survivors.

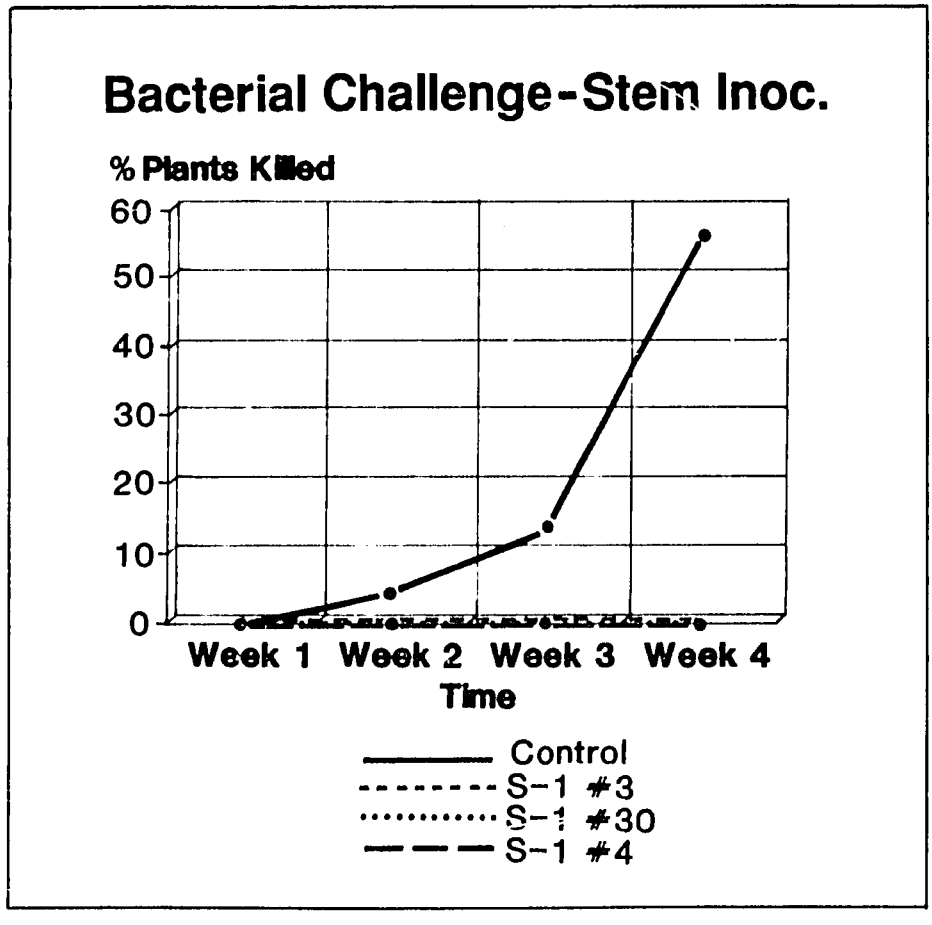


Figure 8. Bacterial challenge experiment. Around 40 F1 seedlings of control and three independent Shiva-1 transformed clones (#3, #4, and #30) were stem-inoculated with about 10^5 *P. solanacearum* bacteria and mortality was evaluated after four weeks. None of the transgenic seedlings died and the symptoms were significantly lower in a scale from 0 (no symptoms) to 5 (dead plants) (data not shown).

Potato Gene Promoters and Their Applications

W. B. Frommer[1], U. Sonnewald[1], A. von Schaewen[2], S. Prat[1],
J. J. Sánchez-Serrano[1], J. Stockhaus[1], and L. Willmitzer[1]

Abstract

A concept is proposed that tries to combine classical breeding and botanical research with the methods of molecular genetics in order to improve the knowledge of such complex characters as crop yield and quality. A better understanding of the mechanisms that guide, e.g., carbohydrate partitioning might be a way to influence crop yield. The concept proposed for potato is based on the possibility of generating molecular mutants by fusing specific promoters to genes coding for certain functions involved in carbohydrate partitioning. This paper gives the results of two applications: the expression of yeast invertase in transgenic plants and the reduction of the water splitting complex of photosystem II-associated 10kD protein in transgenic potato.

Yield characteristics of the potato plant

The potato crop mainly propagates vegetatively via tubers that represent radially expanded internodes derived from underground stems (stolons) (Artschwager, 1929). Tuberization is not restricted to stolons but can be induced by a variety of mechanisms on sprouting tubers or at the axillary buds (Wellensiek, 1924). In field-grown plants, induction of this differentiation process is controlled by a number of environmental conditions such as photoperiod, temperature and nitrogen supply (Ewing, 1985). Tuber-inducing factors have been identified in leaves of both potato and tobacco (Koda et al., 1988).

Plant growth and development require energy obtained through fixation of carbon dioxide into carbohydrates during photosynthesis. The primary sites for photoassimilation are the green parts of a plant, i.e. the leaves and, to a lesser extent, the stem, whereas other organs such as roots, seeds or storage organs like tubers do not contribute to carbon assimilation, but rather are dependent on photosynthetically active organs. The energy from the photosynthetically active tissues, the so-called sources (defined as net exporters of fixed carbon) thus flows to the photosynthetically inactive parts of a plant, the utilization or storage sinks (defined as net importers of fixed carbon).

[1]Wolf B. Frommer, Uwe Sonnewald, Salomé Prat, José J. Sánchez-Serrano, Jörg Stockhaus, and Lothar Willmitzer, Institut für Genbiologische Forschung Berlin GmbH, Berlin, Federal Republic of Germany.
[2]Antje von Schaewen, Dept. of Biology C-016, University of California, San Diego, CA, U.S.A.

During plant development an organ is continuously changing with respect to its state as a sink or a source. Essentially all plant organs at certain stages act as a sink. During plant development, however, the relative sink strength (defined as the ability of a sink to import assimilates) changes (Ho, 1988). During vegetative growth, roots and developing leaves constitute strong sinks, whereas with the onset of flowering the bulk of the assimilate flows into developing seeds, roots and tubers, which represent the strongest sinks during this developmental stage. Sink-source relationships are fundamental for crop yield. They also represent an interesting system for the study of developmental processes.

One example for such a transition are the leaves, which change during their growth from being a semiheterotrophic sink in the young stage to an autotrophic source in their mature stage. Only the mature leaf produces an excess of photoassimilates and is thus able to provide the rest of the plant with energy (Turgeon, 1989). In contrast to many other dicotelydoneous crop species propagating via seeds that use lipids as an energy source for the development of the offspring, potato mainly stores carbohydrates. Whereas in the developing leaf, imported sucrose is utilized for growth and developmental processes, the growing potato tuber represents a strong storage sink in which starch is the major reserve. After the tuber is fully grown it undergoes a period of dormancy. Sprouting is then accompanied by mobilization of storage reserves from the perimedulla (Davies and Ross, 1984). The tuber thus undergoes a sink-to-source transition with little anatomical change. (Fig. 1)

Several models have been described dealing with the central problem of sink-source transitions as well as with the question of what factors determine sink strength. Sucrose is the preferred transport form of carbohydrate in almost all higher plants, and the ability to unload sucrose from the phloem has been considered as an important determinant of sink strength (Ho, 1988). The efficiency of unloading seems to be determined mainly by the maintenance of a concentration gradient between sucrose present in the phloem and the adjacent sink tissue. Both unloading via active transport across membranes and metabolization of sucrose and, thus, removal from the equilibrium, could be the major factors determining relative sink strength.

The use of transgenic plants to study and influence plant gene expression

During recent years, gene transfer into higher plants has become a routine procedure. The technique most frequently applied makes use of a natural plant-directed vector, the gram negative soil bacterium *Agrobacterium tumefaciens*.

Molecular genetics allows the in vitro analysis of the role of single proteins (e.g. enzymes) on metabolism. This is achieved by creating mutants that are deficient in single loci. Mutations that positively influence a trait of interest might be useful for crop improvement. The mutants can either be produced by mutagenesis programs as carried out, e.g., in *Arabidopsis thaliana* (Somerville and Ogren, 1982) or by genetic engineering.

The strategy in genetic engineering is to fuse the coding regions of appropriate genes to different potato promoters and to transfer to plants, thus leading to overexpression, aberrant expression or down regulation of specific proteins. If the gene is fused to a strong promoter this should lead to overexpression of the protein and thus to physiological changes. For aberrant expression in tissues or cell compartments where a protein is usually absent, tissue-specific promoters and/or subcellular targeting signals are fused to genes of interest, plants are transformed, thus leading to expression of the protein in different cell types or subcellular compartments.

In both cases homologous or nonhomologous genes can be used. Heterologous genes can have the advantage if their product underlies a different post-translational regulation as compared to the endogenous enzyme in potato. In vitro mutagenesis of appropriate genes further extends the possibilities.

For the reduction of a specific gene product, an anti-sense RNA under control of appropriate promoters is made. By mechanisms so far not fully understood, the production of sense RNA and its product, can be drastically impaired. The reduction in expression of single genes for gene families permits the analysis of this function and contribution to traits. Furthermore, this approach could be used to eliminate characters negatively influencing a trait.

Characters related to crop yield as subjects for molecular genetics

Yield and its component traits are quantitatively inherited and thus influenced by many genes (Stuber et al., 1987). Several characters that are related to yield are under investigation using the tools of molecular biology e.g. improvement of protein amount and quality, photosynthesis, introduction of the nitrogen fixation pathway, and resistance to pests.

As described above, carbohydrate metabolism in potato is of major importance and thus a critical characteristic to study. By conventional breeding of wheat for example, it was possible to reduce the stem length in favor of a greater allocation of photosynthetic assimilates to the grain filling (Gifford and Evans, 1981).

Prerequisites for such experiments are the availability of a transformation technology and the isolation and analysis of suitable potato promoters and of genes involved in carbohydrate partitioning. Figure 2 gives an overview of several genes and pathways that are important for carbohydrate anabolism, catabolism and partitioning.

We have developed a routine *Agrobacterium tumefaciens*-based transformation system for potato, and have isolated and analyzed several genes that are regulated by environmental, developmental and metabolic signals. The promoters of these genes are able to control the expression of marker genes in transgenic plants. Both cis and trans elements involved in regulation have been analyzed in detail.

The class I patatin gene B33 from potato is strongly expressed in sink tubers and can be induced in leaves by metabolic signals such as sucrose (Rocha-Sosa et al., 1989) whereas the class II patatin gene pGT2 is under tissue-specific control in certain cell types of potato tubers and roots (Köster-Töpfer et al., 1989). The proteinase inhibitor II gene is strongly expressed in sink tubers and inducible by wounding, by ABA and by sucrose (Sanchez-Serrano et al., 1986; Peña-Cortéz et al., 1989).

The ST-LS1 gene is expressed in tissues that contain chloroplasts and thus is expressed in a light-dependent mode in the leaves and stem of potato (Stockhaus et al., 1989).

Furthermore, due to the fact that regulation of several promoters is conserved across species (e.g. Köster-Töpfer et al., 1989), heterologous promoters from dicotyledoneous plants (for a summary see Benfey and Chua, 1989) could serve the same purpose. These promoters can be fused with cDNAs of genes involved, e.g., in carbohydrate metabolism, in sense and antisense orientation and transferred to potato. Analysis of transgenic plants with respect to enzyme activities and metabolism can give an insight into the role and contribution of such genes and could modify the partitioning.

Examples for application

An example for the aberrant expression of a heterologous protein that plays a role in carbohydrate partitioning is invertase, a protein that irreversibly degrades sucrose to glucose and fructose. Chimeric genes consisting of the coding region of the yeast invertase gene suc2 and different N-terminal portions of the potato-derived vacuolar protein proteinase inhibitor II under control of the CaMV 35S promoter, were transferred to tobacco and *Arabidopsis thaliana* plants using *Agrobacterium tumefaciens*-based systems (von Schaven et al., submitted). Regenerated transgenic plants display a new invertase activity absent from untransformed control plants. In vitro transcription/translation experiments in the presence or absence of microsomal fractions and the analysis of transiently or stably transformed cells could show that the products of the different constructs are efficiently taken up into the lumen of the ER. The protein is N-glycosylated and efficiently secreted to the apoplast.

Whereas expression of the invertase leads only to small changes in transgenic *Arabidopsis* plants, transgenic tobacco plants show dramatic changes with respect to development and habit. Expression of the invertase leads to stunted growth, bleached and necrotic regions in leaves and to repressed root formation. These phenotypes are linked to source-sink transitions occurring during plant development. It is assumed that the sucrose which is loaded via active transport into the apoplast in order to enter the phloem is metabolized by the constitutively active invertase present in the apoplast. The hexoses cannot be taken up by the phloem, but must reenter the cell to be resynthesized to sucrose. Both transport and resynthesis are energy-requiring steps so that a futile cycle is generated leading to bleaching and necrosis in the leaves. Due to the energy depletion and the reduced amounts of sucrose available for phloem transport the growth of the plant is

impeded. It will be interesting to analyze the phenotype in potato and to target the protein to different tissues and subcellular positions.

As an example of the reduction of specific proteins via the antisense approach the function of a nuclear gene involved in photosynthesis was investigated. A chimeric gene encoding an antisense RNA of the 10kD protein of the water splitting apparatus of photosystem II of potato under control of the CaMV 35S promoter, was introduced into potato using *Agrobacterium*-based vectors (Stockhaus et al., submitted). The expression of the antisense RNA led to a significant reduction of the amounts of the 10kD protein and RNA in a number of transgenic plants. In 3 out of 36 plants tested, the level of the 10kD protein was only 1-3% compared to the wild type control. The accumulation of other photosystem II-associated polypeptides did not seem to be affected either at the RNA or protein level. Phenotypic differences between wild type potato plants and the constructed mutants with respect to growth rate, habit or ultrastructure of the chloroplasts were not found. However, measurements using isolated thylakoids indicated that the 10kD protein might be involved in stabilizing photosystem II.

Conclusion

Both aberrant expression of invertase and reduction of expression levels of the ST-LS 1 gene described above seem to be practical approaches for the study of plant physiology and development. Future research in this direction might lead to a better understanding of complex traits like carbohydrate partitioning or photosynthetic effectivity. Work on other genes involved in yield-relevant characters is in progress.

References

Artschwager, E. F. 1924. Studies on the potato tuber. J. Agric. Res. 27:809-835.

Benfey, P. N. and N. M. Chua. 1989. Regulated genes in transgenic plants. Science 244:174-181.

Davies, H. V. and H. A. Ross. 1984. The pattern of starch and protein degradation in tubers. Potato Res. 27:373-381.

Ewing, E. 1985. Cuttings as simplified models of the potato plant. *In* P. H. Li (ed.). Potato Physiology. pp. 153-207.

Gifford, R. M. and L. T. Evans. 1981. Photosynthesis, Carbon Partitioning, and Yield. Ann. Rev. Plant Physiol. 32:485-509.

Ho, L. C. 1988. Metabolism and Compartmentation of imported Sugars in Sink Organs in Relation to Sink Strength. Ann. Rev. Plant Physiol. Plant Mol. Biol. 39:355-378.

Koda, Y., E. A. Omer, T. Yoshida, H. Shibata, S. Sakamura, and Y. Okazawa. 1988. Isolation of a potato tuber-inducing substance from potato leaves. Plant Cell Physiol. 29:1047-1051.

Köster-Töpfer, M., W. B. Frommer, M. Rocha-Sosa, S. Rosahl, J. Schell, and L. Willmitzer. 1989. A class II patatin promoter is under developmental control in both transgenic potato and tobacco plants. Mol. Gen. Genet. 219:390-396.

Pena Cortéz, H., J. J. Sánchez-Serrano, R. Mertens, L. Willmitzer, and S. Prat. 1989. Abscisic acid is involved in the wound-induced expression of the proteinase inhibitor II gene in potato and tomato. Proc. Natl. Acad. Sci U.S.A. 86:9851-9855.

Rocha-Sosa M., U. Sonnewald, W. B. Frommer, M. Stratmann, J. Schell, and L. Willmitzer. 1989. Both developmental and metabolic signals activate the promoter of a class I patatin gene. EMBO J. 8:23-29.

Sánchez-Serrano, J. J., S. Schmidt, J. Schell, L. Willmitzer. 198[illegible]. Nucleotide sequence of Proteinase inhibitor II encoding cDNA of potato (*Solanum tuberosum*) and its mode of expression. Mol. Gen. Genet. 203:15-20.

Somerville, C. R. and W. L. Ogren. 1982. Isolation of photorespiration mutants in *A. thaliana*. *In* M. Edelman, R. Hallick, and N. H. Chua (eds.). Methods in Chloroplast Molecular Biology, Elsevier New York. pp. 129-138.

Stockhaus, J., J. Schell, L. Willmitzer. 1989. Correlation of the expression of the nuclear gene ST-LS1 with the presence of chloroplasts. EMBO J. 8:2445-2451.

Stockhaus, J., M. Höfer, G. Renger, P. Westhoff, T. Wydrzynski, and L. Willmitzer. Transgenic potato plants devoid of the 10kD polypeptide of photosystem II due to inhibition by anti-sense RNA are phenotypically indistinguishable from wild type plants. EMBO J. (submitted).

Stuber, C. W., M. D. Edwards, J. F. Wendel. 1987. Molecular marker facilitated investigation of quantitative trait loci in maize: II Factors influencing yield and its component traits. Crop. Sci. 27:639-648.

Turgeon, R. 1989. The sink source transition in leaves. Ann. Rev. Plant Physiol. Plant Mol. Biol. 40:119-138.

Von Schaewen, A., S. Schmidt, U. Sonnewald, and L. Willmitzer. Apoplastic expression of a yeast derived invertase strongly influences development and habitus of transgenic tobacco plants linked to source sink transitions. EMBO J. (submitted).

Wellensiek, S. 1929. The physiology of tuber formation in *Solanum tuberosum*. Meded. Landbouwhogeschool Wageningen 33: 6-4.

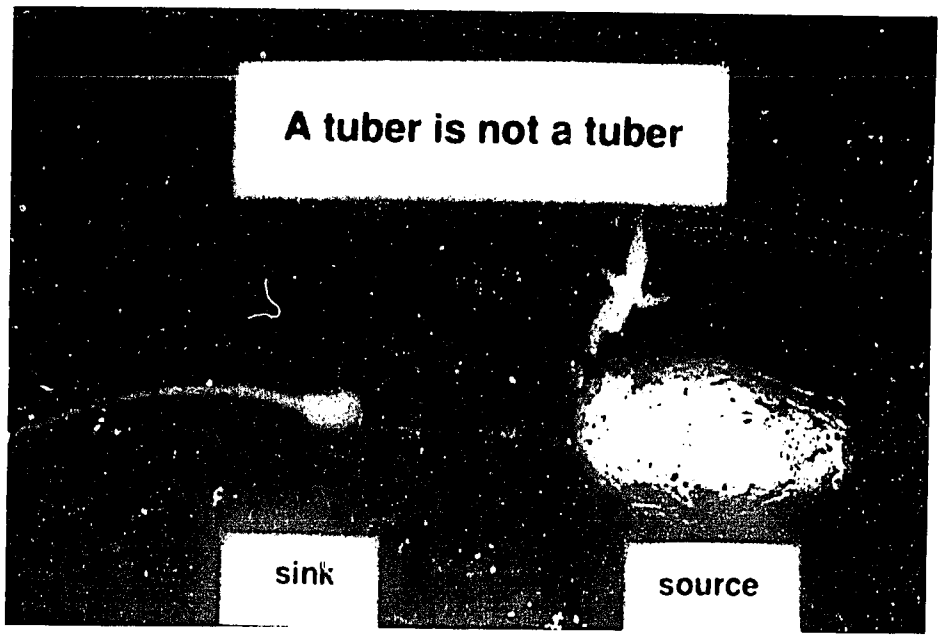


Figure 1. Sink-to-source transition in potato tubers. The potato tuber switches from a sink when it develops to a source when it starts to sprout.

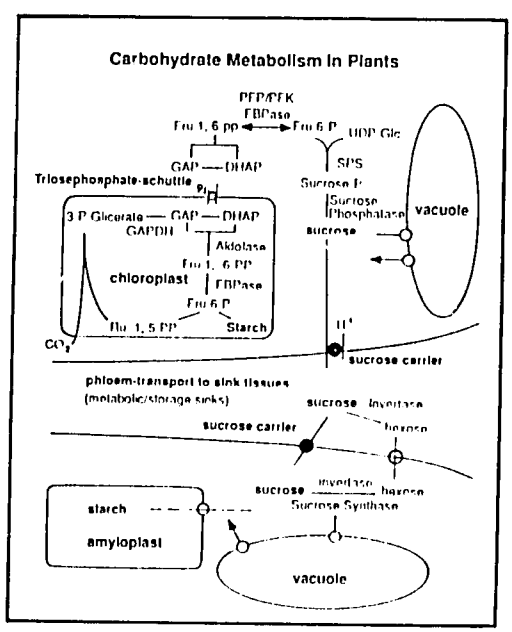


Figure 2. Carbohydrate flow in source and sink. CO_2 is fixed in the chloroplasts of the leaf and transiently stored in form of starch. Carbohydrates are transported in form of trioses via an active carrier to the cytoplasm, where in several steps sucrose is formed. A pool of sucrose is present in the vacuole. Sucrose is actively translocated to the phloem, and transported via mass flow to the various sinks. Actively and/or passively sucrose enters the sink cells and is used for metabolism or storage in form of starch.

Coat Protein-Mediated Resistance: A New Type of Resistance to Control Plant Viruses

C. Fauquet and R. N. Beachy[1]

Profile

Coat protein-mediated resistance is a new type of resistance to plant viruses, resulting from the interaction of the coat protein (CP) constitutively expressed in the cells of transgenic plants with virus replication. After isolating the CP gene from the virus genome, it is ligated with sequence elements for gene expression, the chimeric gene is introduced into plant cells, and transgenic plants are regenerated. This methodology has been applied successfully to nine viruses belonging to seven virus groups in four different plants, including potato. Potato is affected by many viruses belonging to 15 virus groups, including five of the seven virus groups for which coat protein-mediated resistance has been achieved. Transgenic potatoes resistant to potato virus X and potato virus Y were produced and a transgenic line was shown to be highly resistant to both viruses. These plants were also resistant to PVY by aphid inoculation and no PVX particles could be recovered from tubers of infected transgenic plants. The specificity of the coat protein-mediated resistance is broader than "natural" virus resistance and can protect against virus infection for viruses differing up to at least 40% in their coat protein sequences. Coat protein-mediated resistance is shown to be a multi-component type of resistance, exhibiting resistance to virus infection, virus multiplication, symptom expression, and virus spread. Because in most of the cases the coat protein-mediated resistance can be overcome by viral RNA inoculation, it has been envisaged that this mechanism could be related to virus decapsidation, but this remains to be proven.

Introduction

The development of the tools of plant genetic engineering has made it possible to introduce chosen and selected genes into plants to confer novel phenotypes and new interesting traits. Some genes are introduced for their potential agricultural application in herbicide resistance, insect resistance, and virus resistance.

In the last few years, there have been a number of examples of genetic modification of plants to produce virus disease resistance in transgenic plants. However, until now, only the coat protein-mediated resistance has provided several examples of engineered resistance for several

[1]Claude Fauquet and R. N. Beachy, Washington University, Dept. Biology, Missouri Mo, U.S.A

plants and different viruses. This type of resistance is stable and sufficiently established to predict that in the future, resistant plants could be cultivated in many countries.

The principle of this resistance, described in the following sections, is very simple and theoretically applicable to all the plant viruses, thereby providing new possibilities for deriving virus resistance in many different plants. To date, seven different virus groups have been successfully tested with this new technology, for five different plants including potato, and so far none have shown negative results. We will briefly review the results obtained for viruses belonging to virus groups whose members infect potato, and especially the results obtained with transgenic potatoes.

The value of CP-mediated resistance has been studied in detail in a limited number of cases, nevertheless, we now have evidence showing that it is of a multi-component resistance type. The parameters of resistance have been investigated most extensively with the tobacco mosaic virus, and we will give a summary of the information accumulated in terms of specificity of the protection and type of resistance.

The precise mechanisms for CP-mediated resistance are not fully understood and it is likely that a better understanding of these mechanisms will lead to improvement in the resistance.

Coat Protein-Mediated Resistance

Definition

Coat protein-mediated resistance refers to the resistance to virus infection caused by expression of a coat protein (CP) gene in transgenic plants. The expression of a CP gene confers resistance to the virus from which the CP gene was derived and to related viruses. Resistance is not due to somaclonal variation caused by the transformation event or the tissue culture procedure, and is stably inherited by subsequent generations.

The Mechanics of Producing Coat Protein-mediated Resistance

Producing virus-resistant plants requires the knowledge of a number of important characters of a system, none of which can be considered more important than the next. For example, it is imperative that the genomic structure and organization of the virus are understood, thereby enabling the isolation of the CP open reading frame of the virus.

Construction of a chimeric gene will include the selection of an appropriate transcriptional promoter to cause the expression of the CP gene at sufficient levels and with sufficient tissue specificity to produce disease resistance. Several different transcriptional promoters have been used and the promoter that has proven most effective is derived from cauliflower mosaic virus (CaMV) and is responsible for transcribing the 35S RNA transcript during CaMV infection. This promoter, P35S, leads to high levels of mRNA and protein in most of the plants in which it has

been tested. Furthermore, an enhanced 35S promoter, PE35S, which is produced by duplication of an upstream regulatory sequence (Kay et al., 1987), causes a higher level of gene expression.

The role of non-translated sequences in gene expression should also be considered during gene construction. Powell et al. (1990) found that deleting the sequences 3' of the termination codon of the TMV CP gene increased the level of CP accumulation while not affecting the level of mRNA. Similar experiments were done during construction of the SMV CP gene (Stark, 1989). However, deleting the 3' non-translated region of the RNA had little or no effect on accumulation of either mRNA or CP.

The third part of the chimeric genes is a sequence to confer transcript termination and polyadenylation. Little evidence has been published to date indicating that a specific 3' end is preferred in transgenic plants. The 3' ends used for most chimeric genes expressed in plants have been taken from CaMV, a variety of genes in the T-DNA region of the Ti plasmid, storage proteins, and genes that are regulated by light. Powell et al. (1986) suggested that read-through of the NOS 3' end on the TMV CP gene was responsible for multiple sizes of CP mRNA in tobacco plants. On the other hand, expression of the same CP gene in tomato plants led to the accumulation of mRNAs of the predicted size (Nelson et al., 1988). Replacement of the NOS 3' end on the TMV CP gene with the 3' end from a seed storage protein led to accurate processing of the transcript in tobacco plants (Powell et al., 1990).

The phenotype of Coat Protein-Mediated Resistance

In order to use populations of plants that are identical in age, growth rate, and size, R1 or successive generations of plants, regenerated after transformation to introduce the CP genes, are generally used. Prior to inoculating seedlings with virus, the segregation of the introduced gene must be determined. This can be conveniently done by producing an immunological reaction to detect the CP or by following the expression of a gene that is co-introduced with the CP gene.

Assessing disease resistance involves inoculating with virus plants that express the CP Gene CP(+) and those that do not CP(-). A comparison in the numbers of sites of infection, the disease incidence, the development of disease symptoms, and the accumulation of virus in CP(+) and CP(-) plants is generally used to evaluate resistance.

Viruses infecting potato

Throughout the world, potatoes are infected by more than 20 different viruses (de Bokx et al., 1987; Hooker, 1981) belonging to 15 virus groups. The importance of each virus in terms of spread and economic impact varies according to geographical area and the potato cultivar. Table 1 presents a list of the most important potato viruses in the world, as well as those in the Andean regions.

Potato viruses are not only important because of the yield losses that they cause, but also because most of the viruses are transmitted through the tubers allowing their spread in the new

fields and between countries. Furthermore, synergistic effects between different viruses, especially PVX, have frequently been observed in potato fields. Many genes for resistance to several viruses have been found and transferred successfully to potato cultivars (de Bokx et al., 1987). Nevertheless, viruses remain a constant problem for growers because the selected resistant potato cultivars are not always acceptable to the consumers. Extensive (and expensive) certification programs have been set up in many countries in order to decrease the spread and impact of potato viruses.

From the biotechnology perspective, potato is particularly interesting because of the number of viruses belonging to different groups which infect it and because the potato is an easy plant to regenerate after transformation (Ooms et al., 1987; Ooms et al., 1983; Ooms et al., 1985; Sheerman et al., 1988). The CP-mediated resistance strategy has been successfully demonstrated with members of 6 out of the 15 virus groups infecting potatoes, and several transgenic potato cultivars have already been positively tested for their resistance against two viruses (Hoekema et al., 1989; Lawson et al., 1990). Because the potato is vegetatively propagated from the virological point of view, it is interesting to assess the impact of such resistance on the transmission of viruses through tubers.

Examples of coat protein-mediated resistance

Since 1986, there have been a number of reports of CP-mediated resistance involving a variety of different viruses and different host plants. A list of published and unpublished reports, is presented in Table 2. The list includes viruses in seven different groups and hosts that include members of the *Solanaceae* and *Leguminosae*. We will review here only the results obtained for the most important viruses of potato, but complementary information can be found in other reviews on the CP-mediated resistance (Beachy et al., in preparation).

Potexvirus group: Potato Virus X (PVX)

Chimeric genes were introduced via disarmed strains of *Agrobacterium tumefaciens* into tobacco (Hemenway et al., 1988) or potato (Hoekema et al., 1989; Lawson et al., 1990). Regenerated potato lines included the commercially important cultivars Bintje and Escort (Hoekema et al., 1989), and Russet Burbank (Lawson et al., 1990). Regenerated potato and tobacco plant lines accumulated PVX CP at the level of 0.05 to 0.3% (w/w) of total plant protein. Resistance to PVX was tested in potato plants derived from rooted cuttings. Each of the CP(+) potato cultivars accumulated much less virus (20 to 50 fold less) after inoculation with PVX than did CP(-) plant lines, and CP(+) lines with higher levels of CP were more resistant than lines with lower levels of CP (Hoekema et al., 1989; Lawson et al., 1990; data not shown).

Resistance was assessed on tobacco leaves by quantitatying the numbers of infection sites (Hemenway et al., 1988). On one plant line the number of lesions on CP(+) plants was 10-15% of the lesions on CP(-) plants. While 100% of the CP(-) plants developed symtoms by day 11, between 10% and 30% of the CP(+) plants developed symptoms by day 18. Furthermore, there

was a correlation between the level of CP in transgenic plants and the degree of resistance (Hemenway et al., 1988).

It had been reported that mediated resistance against TMV (Nelson et al., 1987) and ALMV (Loesch-Fries et al., 1987) was largely overcome by inoculation with viral RNA. However, the relative number of lesions produced by PVX RNA on CP(+) plants was essentially equivalent to that produced by PVX (Hemenway et al., 1988). These results may indicate that the mechanism(s) of CP-mediated resistance against PVX is different than resistance against other viruses.

Dual protection against PVX and PVY

Lawson (1990) isolated a cloned cDNA encoding the CP of PVY, and modified the clone to contain an AUG initiation codon, and the gene was introduced into Russet Burbank potato. Several plant lines contained PVY CP equivalent to 0.01-0.05% (w/w) of potato leaf protein (Lawson et al., 1990). In this study, a gene encoding PVX CP was also introduced on the plasmid with the PVY CP gene and led to the accumulation of PVX CP at levels of 0.05-0.2% (w/w) of total leaf protein.

Rooted cuttings of plant lines carrying only the PVX gene or both PVX and PVY CP genes were mechanically inoculated with either PVX, PVX plus PVY, or PVY alone. All plant lines tested were resistant to infection by PVX, and tubers recovered from the resistant inoculated plants did not contain PVX. The CP(-) plants were readily infected under these conditions and virus accumulated to high levels in both leaves and tubers (Lawson et al., 1990). As expected, plant lines harboring only the PVX gene were susceptible to PVY.

When plant lines carrying both PVX and PVY CP genes were inoculated with PVY, each line showed resistance to infection under inoculum concentrations (20 μg/ml) in which 80% of CP(-) plant lines became infected. Four plant lines that express both genes were also doubly inoculated with PVX (5 μg/ml) and PVY (20 μg/ml). Each was more resistant to PVX than the CP(-) lines. One line was highly resistant to both PVX and PVY in all experiments, and in most cases escaped infection (Lawson et al., 1990).

Resistant plant lines were also inoculated with PVY via green peach aphids, and though the efficiency of infection was low, two CP(+) lines were somewhat resistant to PVY. However, by 35 d.p.i. these lines were not different from CP(-) lines in levels of infection. The highly resistant line, described earlier, did not become infected with PVY in the aphid transmission studies (Lawson et al., 1990). It is interesting to note that this line contains less PVX and PVY CP than other lines. The authors suggest that other unidentified factors, in addition to CP gene expression, can influence levels of resistance.

Potyviruses: Potato virus Y (PVY) and Tobacco etch virus (TEV)

In another study (Stark et al., 1989), a gene encoding a CP from soybean mosaic virus (SMV) strain N (Eggenberger et al., 1989) was introduced into *Nicotiana Tabacum* Xanthi (SMV is not a pathogen of tobacco). The CP cDNA was altered to place the AUG initiation codon in a consensus sequence favorable for efficient translation (Lutke et al., 1987). A number of transgenic plant lines harboring the SMV CP gene were produced with SMV CP levels ranging from non-detectable to 0.23% (w/w) of extractable protein (Stark et al., 1989). Multiple lines were inoculated with tobacco etch virus (TEV) and potato virus Y (PVY), which are antigenically distinguished from each other and from SMV. Comparisons of the amino acid sequences of the CP of each virus show 55 to 60% homology among the three (Stark et al., 1989). While a number of plant lines show low levels of resistance to both TEV and PVY (Stark, 1989), one line was highly resistant to inoculum levels ranging from 0.5 μg/ml to 50 μg/ml. When this line was made homozygous with respect to the SMV CP gene, plants were not infected with either virus at these inoculum levels.

Stark et al. (1989) also used a disease rating system to quantity symptoms on systemically infected plants. Although many CP(+) plants escaped infection, others became infected, yet developed very mild symptoms on one or several leaves by 12 d.p.i. Low disease ratings correlated with low levels of virus replication.

CP-mediated resistance against potyviruses has several important features. First, it is possible to produce plant lines that are highly resistant or immune to infection. Second, at least one CP(+) plant line is highly resistant to inoculation by viruliferous aphids. Third, a CP gene from a potyvirus that is not a pathogen (SMV) can protect transgenic tobacco plants against pathogenic potyviruses (PVY, TEV). This is referred to as "protection against heterologous viruses". Fourth, while CP accumulation leads to high levels of resistance in some lines, other CP(+) lines have very poor or no resistance to infection. The differences between the highly resistant and poorly resistant lines have not yet been determined.

Tobraviruses: Tobacco rattle virus (TRV)

A TRV CP gene was constructed with the CP cDNA from TRV strain TMC, (Angement et al., 1986; van Dun et al., 1987). The chimeric gene was introduced into *N. tabacum* cv. Samsun NN. The CP(+) plant line, which contains 0.05% (w/w) TRV CP, produced few, if any, symptoms after inoculation with 1 μg/ml TRV strain TCM (van Dun et al., 1988). CP(+) plant lines also accumulated little or no TRV RNA.

This plant line was also inoculated with TRV strain PCB and pea early browning virus (PEBV). Strain PCB has approximately 39% amino acid sequence homology with strain TCM; the PEBV isolate has considerably more homology with the TCM strain (Angement et al., 1986). This line was not resistant to TRV-PCB, while inoculation with PEBV caused 75% fewer lesions than on CP(-) plants. Van Dun et al. (1988) concluded that resistance to PEBV reflected the high degree of relatedness of the PEBV and TRV-TCM coat proteins, which interferes with infection

by PEBV as well as TRV-TCM. It is unlikely, however, that the interference is at the level of CP-RNA interactions since CP molecules of each strain are able to encapsulate the RNAs of the other.

Evaluation of the coat protein-mediated resistance

Specificity of the CP-mediated resistance

A study on the spectrum of resistance of transgenic plants expressing a CP gene was carried out by Nejidat et al. (1990) and Anderson et al. (1989). In these studies CP(+) tobacco plant lines that expressed the U_1 TMV CP gene were inoculated with other tobamoviruses. Based upon comparisons of amino acid sequences and/or composition of virus CPs (Gibbs, 1986), tomato mosaic virus (ToMV), pepper mild mottle virus (PMMV), tobacco mild green mosaic virus (U_2 strain of TMV; TMGMV), Odontoglossum ringspot virus (ORSV), ribgrass mosaic virus (RMV) and sunn hemp mosaic virus (SHMV) have degrees of relatedness to TMV ranging from 85% homology (ToMV) to 39% homology (SHMV). Infections by TMV, ToMV, PMMV, and TMGMV were inhibited by 95-98%, ORSV by 80-95%, RMV and SHMV by 40-60% (Nejidat et al., 1990). Furthermore, systemic spread of infection in CP(+) Xanthi tobacco (when it occurred) was significantly delayed in CP(+) plant lines. On the basis of these studies, it was concluded that viruses that are related to the CP of TMV by more than 50-60% are less able to infect the resistant lines than are less related tobamoviruses.

Anderson et al. (1989) also inoculated TMV CP(+) plant lines with members of different virus groups. Tobacco plants that contain TMV CP were inoculated with cucumber mosaic virus (CMV), Alfalfa mosaic virus (ALMV), PVX, and PVY. There was no resistance against infection by any of the viruses on inoculated leaves of CP(+) plants; however, there were somewhat reduced rates of systemic spread of CMV, PVX, and PVY in CP(+) compared with CP(-) plants.

As for the potyviruses, the experiment of Stark et al. (1989) documents a high degree of protection between viruses having approximately 60% homology in CP sequence (see details above). Heterologous protection with potyviruses having less homology has not yet been done. In the case of tobraviruses, the heterologous protection is also effective for viruses having about 60% homology (see details above).

Multiple manifestations of resistance

Resistance to inoculation. In each of the examples of CP-mediated resistance described to date, resistance is manifested by several features. First, there is a reduction in the number of sites where infection occurs on inoculated leaves. Hemenway et al. (1988) reported that fewer lesions were produced after inoculation with PVX on CP(+) tobacco plants that on CP(-) plants. Powell et al. (1986) reported fewer chlorotic lesions caused by TMV infection on tobacco plants that express the TMV CP gene than on those that did not. Likewise, Nelson et al., (1987) found that the numbers of necrotic local lesions caused by TMV infection on CP(+) Xanthi tobacco local lesion were 95 to 98% lower than on CP(-) plants. These experiments indicate that the expression

of a CP gene causes a reduction in the number of sites where infection is established upon inoculation.

Resistance to symptom expression. A second manifestation of resistance is a reduced rate of disease development on systemic hosts that are CP(+). In each of the examples of CP-mediated resistance, CP(+) plant lines were less likely to develop systemic disease symptoms than those that were CP(-). Thus, if inoculation results in an infection on the inoculated leaves, the likelihood that the infection will become systemic is considerably lower in plants that are CP(+) compared with those that are CP(-). Hemenway et al. (1988) reported that several plant lines that expressed the PVX CP gene did not become systemically infected when inoculated with high levels of virus. Similar results were reported for CP-mediated resistance against CCMV (Cuozzo et al., 1988), TMV (Powell et al., 1986), and other viruses.

Resistance to virus multiplication. A third manifestation of resistance is lower accumulation of virus in CP(+) compared with CP(-) plant lines. ELISA and semi-quantitative western-or immuno-dot blots have been used to quantitate virus accumulation in inoculated leaves and other plant parts in most examples of CP-mediated resistance. In certain examples of resistance, plants accumulate little or no virus after inoculation. Such examples can be considered to be immune to infection under the tests conditions.

Resistance to virus spread within the plant. Wisniewski et al. (1990), carried out a grafting study in which a stem segment of a transgenic tobacco TMV (CP+) was inserted between the rootstock and apex of a wildtype tobacco. When a hybrid plant was inoculated on the lower leaves, the CP(+) segment prevented the virus from moving to the upper part of the grafted plant. This experiment shows that the CP may play a role in the long-distance movement of the virus and consequently, resistance has a component that affects systemic spread of the infection, at least in the association TMV-tobacco.

Understanding the mechanisms of CP-mediated resistance

From the descriptions of CP-mediated resistance presented, it is clear that there are differences between different viruses. The unifying observation above is that CP molecules are responsible for resistance (Powell et al., 1986; van Dun et al., 1988). The question of how the CP confers resistance remains unclear, and the nature of virus infection and disease development makes it likely that resistance is affected at several stages.

Upon introduction of a virus into its host, either by mechanical wounds, or by vector transmission, the initial step in infection involves the release of the nucleic acid for translation by host ribosomes. It has been proposed that for many, if not all, viruses swelling of the particle precedes the release of the nucleic acid (Wilson, 1985). Upon swelling and release of the 5' end of the RNA, binding of ribosomes and disassembly of the virus concurrent with translation initiates the infection process. Does CP interfere in some way with this process?

As described above, CP-mediated resistance reduces the number of sites of infection on infected leaves. Several types of experiments indicate that CP gene expression interferes in some way with an early event in infection. First, resistance can be overcome in some but not all examples, by inoculation with viral RNA rather than virions. (Clemenway et al., 1988; Loesch-Fries et al., 1987; Nelson et al., 1987; Powell et al., 1986; van Dun et al., 1987).

Because studying "early events" in virus replication are, at best, imprecise in whole leaves, studies with protoplasts are preferred when possible. Two examples of CP-mediated resistance have been studied with tobacco protoplasts. Loesch-Fries et al. (1987) reported resistance against infection of ALMV CP(+) protoplasts with ALMV, but not ALMV RNA. Likewise Register et al.(1988) reported less resistance against TMV RNA than against TMV in protoplasts isolated from TMV CP(+) plant lines. In the same study, pre-swelled TMV also overcame CP-mediated resistance in protoplasts and in leaves.

Since the release of viral RNA from virus particles is an early step in infection, the results of these experiments support the hypothesis that CP interferes with an early event in infection that releases the encapsidated RNA. This does not necessarily eliminate a role for CP in binding to viral RNA per se to either block uncoating, or gene expression. However, the example of CP-mediated protection with TRV also speaks against a protein-RNA role in resistance (van Dun et al., 1988). The result, along with lack of resistance against viral RNA and pre-swelled virus particles, argues against re-encapsidation of viral RNA as a primary mechanism of resistance. Whether this will be a common feature of CP-mediated resistance remains to be seen.

Conclusion

Coat protein-mediated resistance has proven to be an effective strategy against several viruses in different plants, including potato. Furthermore, the dual resistance to PVX and PVY in potatoes demonstrates the application of the technique to an important food crop. The range of coat protein-mediated resistance specificity is broad, and one CP gene can provide protection against viruses having more than 60% homology between their CP sequences. This new type of resistance has many components: the CP interferes with virus infection, virus multiplication, symptom expression, and the long-distance spread of the virus within the plant. An important consequence for vegetatively propagated plants is the fact that PVX could not be covered from the tubers in resistant transgenic potatoes. The coat protein-mediated resistance is a useful technology to confer virus resistance to a particular potato cultivar and to increase yield.

References

Anderson, E. J., D. M. Stark, E. N. Nelson, and R. N. Beachy. 1989. Transgenic plants that express the coat protein gene of TMV or ALMV interfere with disease, development of non-related viruses. Phytopathology, 12:1284-1290.

Angement, G. C., H. J. M. Linthorst, A. F. Van Belkum, B. J. C. Cornelissen and J. F. Bol. 1986. RNA2 of Tobacco rattle virus strain TMC encodes an unexpected gene. Nucleic Acids Research, 167:4673-4682.

Cuozzo, M., K. M. O'Connell, W. Kaniewshi, R. X. Fang, N. H. Chua, and N. E. Tumer. 1988. Viral protection in transgenic tobacco plants expressing the cucumber mosaic virus coat protein or its antisense RNA. Bio-Technology, 6:549-557.

de Bokx, J. A. and J. P. H. van der Want. 1987. Viruses of potatoes and seed-potato production (2nd ed.). Wageningen: Pudoc, 259 pp.

Eggenberger, A. L., D. M. Stark and R. N. Beachy. 1989. Nucleotide sequence and expression of the SMV coat protein region in *Escherichia coli* and tobacco callus. Journal of General Virology, 70:1853-1860.

Gibbs, A. 1986. Tobamovirus classification. *In* M. H. V. van Regenmortel and H. Fraenkel-Conrat (eds.), the Plant Viruses: Vol. 2 the Rod-shaped Plant Viruses. New York, Plenum Press. pp. 168-180.

Hemenway, C., R. X. Fang, W. K. Kaniewshi, N. H. Chua, and N. E. Tumer (1988). Analysis of the mechanism of protection in transgenic plants expressing the potato virus X coat protein or its antisense RNA. EMBO, 7:1273-1280.

Hoekema, A., M. J. Huisman, L. Molendijk, P. J., M. van den Elzen and B. J. C. Cornelissen. 1989. The genetic engineering of two commercial potato cultivars for resistance to potato virus X. Bio-Technology, 7:273-278.

Hooker, W. J. 1981. Compendium of potato diseases. American Phytopathological Society, 259 pp.

Kay, R., A. Chan, M. Daly, and J. McPherson. 1987. Duplication of CaMV 35S promoter sequences creates a strong enhancer for plant genes. Science, 236:1299-1302.

Lawson, C., W. Kaniewski, L. Haley, R. Rozman, C. Newell, and N. T. Sanders. 1990. Engineering resistance to mixed virus infection in a commercial potato cultivar: resistance to potato virus X and potato virus Y in transgenic Russet Burbank. Bio-Technology, 8(2):127-134.

Loesch-Fries, L. S., D. Merlo, T. Zinnen, L. Burhop, K. Hill, K. Krahn, N. Jarvis, S. Nelson, and E. Halk. 1987. Expression of alfalfa mosaic virus RNA4 in transgenic plants confers virus resistance. EMBO Journal, 6:1845-1851.

Lutke, H. A., K. C. Chow, F. S. Mickel, K. A. Moss, H. F. Kern, and G. A. Scheele. 1987. Selection of AUG codons differs in plants and animals. EMBO Journal, 6:43-48.

Nejidat, A. and R. N. Beachy. 1990. Transgenic Tobacco Plants expressing a Tobacco Virus cort protein gene are resistant to some Tobamoviruses. Molecular Plant Microb Interaction (in press).

Nelson, R. S., S. M. McCormick, X. Delannay, P. Dubé, J. Layton, E. J. Anderson, M. Kaniewska, R. K. Proksch, R. B. Horsch, S. G. Rogers, R. T. Fraley, and R. N. Beachy. 1988. Virus tolerance, plant growth, and field performance of transgenic tomato plants expressing coat protein from tobacco mosaic virus. Bio-Technology, 6:403-409.

Nelson, R. S., A. P. Powell, and R. N. Beachy. 1987. Lesions and virus accumulation in inoculated transgenic tobacco plants expressing the coat protein gene of tobacco mosaic virus. Virology, 158:126-132.

Ooms, G., M. M. Burrell, A. Karp, M. Bevan and J. Hille. 1987. Genetic transformation in two potato cultivars with T-DNA from disarmed *Agrobacterium*. Theoretical Applied Genetics, 73:744-750.

Ooms, G., A. Karp, and J. Roberts. 1983. From tumor to tuber; tumor cell characteristics and chromosome numbers of crown gall-derived tetraploid potato plant (*Solanum tuberosum* cv. maris Bard). Applied Genetics, 66:169-172.

Ooms, G. and J. R. Lenton. 1985. T-DNA genes to study plant development: precocious tuberisation and enhanced cytokinins in *A. tumefaciens* transformed potato. Plant Molecular Biology, 5:205-212.

Powell, A. P., R. S. Nelson, B. De, N. Hoffmann, S. G. Rogers, R. Fraley, and R. N. Beachy. 1986. Delay of disease development in transgenic plants that express the tobacco mosaic virus coat protein gene. Science, 232:738-743.

Powell, P. A., D. M. Stark, P. Sander, and R. N. Beachy. 1990. Protection against tobacco mosaic virus in transgenic plants that express TMV antisense RNA. Proceedings of the National Academy of Sciences of the United States of America (in press).

Register, J. C. I. and R. N. Beachy. 1988. Resistance to TMV in transgenic plants results from interference with an early event in infection. Virology, 166:524-532.

Sheerman, S. and M. W. Bevan. 1988. A rapid transformation method for *Solanum tuberosum* using binary *Agrobacterium tumefaciens* vectors. Plant Cell Reports, 7:13-16.

Stark, D. M. 1989. Protection against potyvirus infection in transgenic plants: Evidence for broad spectrum resistance. Bio-Technology, 7:1257-1262.

van Dun, C. M. P. and J. F. Bol. 1988. Transgenic tobacco plants accumulating tobacco rattle virus coat-protein resist infection with tobacco rattle virus and pea early browning virus. Virology. 167:649-652.

van Dun, C. M. P., J. F. Bol, and L. van Vloten-Doting. 1987. Expression of alfalfa mosaic virus and tobacco rattle virus coat protein genes in transgenic tobacco plants. Virology, 159:299-305.

Wilson, T. M. A. 1985. Nucleocapsid disassembly and early gene expression by positive strand RNA viruses. Journal of General Virology, 66:1201-1207.

Wisniewski, L. A., P. A. Powell, R. S. Nelson, and R. N. Beachy. 1990. Local and systemic movement of tobacco mosaic virus (TMV) in tobacco plants that express the TMV coat protein gene., submitted.

Table 1. List of the most important viruses infecting potato throughout the world or Andean regions.

Virus name	virus group	vectors type	location
Potato leafroll virus (PLRV)	Luteovirus	Aphids	Worldwide
Potato virus Y (PVY)	Potyvirus	Aphids	Worldwide
Potato virus A (PVA)	Potyvirus	Aphids	Worldwide
Potato virus M (PVM)	Carlavirus	Aphids	Worldwide
Potato aucuba mosaic virus (PAMV)	Potexvirus	Aphids	Worldwide
Potato virus X (PVX)	Potexvirus	Contact	Worldwide
Potato virus S (PVS)	Carlavirus	Contact	Worldwide
Alfalfa mosaic virus (ALMV)	ALMV group	Aphids	Worldwide
Tobacco rattle virus (TRV)	Tobravirus	Nematodes	Worldwide
Tomato spotted wilt virus (TSWV)	TSWV group	Thrips	Tropics
Andean potato mottle virus (APMV)	Comovirus	Contact	Andes
Andean potato lattent virus (APLV)	Tymovirus	Beetle	Andes
Potato virus T (PVT)	?	-	Andes

Table 2. Examples of coat protein-mediated resistance in transgenic plants.

CP Sequence[a]	Transgenic plant	Virus resistance	Reference
TMV	tobacco	TMV	Powell Abel et al., 1936
TMV	tobacco	ToMV	Nelson et al., 1988
TMV	tobacco	PMMV	Nejidat and Beachy, 1990
TMV	tobacco	TMGMV	Nejidat and Beachy, 1990
TMV	tobacco	RMV	Nejidat and Beachy, 1990
TMV	tobacco	ORSV	Nejidat and Beachy, 1990
TMV	tomato	TMV	Nelson et al., 1988
TMV	tomato	ToMV	Nelson et al., 1988
ToMV	tomato	ToMV	Kaniewski et al., unpublished
PVX	tobacco	PVX	Hemenway et al., 1987
PVX	potato	PVX	Lawson et al., 1990
PVY	potato	PVY	Lawson et al., 1990
SMV	tobacco	PVY	Stark and Beachy, 1989
SMV	tobacco	TEV	Stark and Beachy, 1989
ALMV	tobacco	ALMV	Loesch-Fries et al., 1987
ALMV	tobacco	ALMV	Tumer et al., 1987
ALMV	tobacco	ALMV	van Dun et al., 1987
ALMV	tomato	ALMV	Tumer et al., 1987
CMV	tobacco	CMV	Cuozzo et al., 1987
CMV	tomato	CMV	Cuozzo et al., 1987
TRV	tobacco	TRV	van Dun and Bol, 1988
TRV	tobacco	PEBV	van Dun and Bol, 1988
TSV	tobacco	TSV	van Dun et al., 1987

[a]**Abbreviations:**

ALMV = alfalfa mosaic virus
CMV = cucumber mosaic virus
ORSV = *odontoglossum* ringspot virus
PEBV = pea early browning virus
PMMV = pepper mild mosaic virus
PVX = potato virus X
PVY = potato virus Y
RMV = ribgrass mosaic virus
TEV = tobacco etch virus
TMGMV = tobacco mild green mosaic virus
TMV = tobacco mosaic virus
ToMV = tomato mosaic virus
TRV = tobacco rattle virus
TSV = tobacco streak virus

Transformation of Potato (*Solanum tuberosum* L.) Leaf Disc Using *A. tumefaciens*-Mediated Transfer of DNA Sequences Coding for Lytic Peptides*

S. Trinca[1], C. De Pace[1], R. Caccia[1], G. Scarascia Mugnozza[1], J. H. Dodds[2], and J. Jaynes[3]

Key words: *Agrobacterium tumefaciens,* leaf discs, lytic peptides, potato, shoot regeneration, transformation

Profile

Two transformation procedures of potato (*Solanum tuberosum* L., cv.Désirée 2n=4x=48) leaf discs were compared. The aim was to obtain transgenic potato plants whose genome contain novel DNA sequences transferred through *A. tumefaciens*. The *A. tumefaciens* strains used contained a disarmed plasmid harboring the reporter genes expressing B-glucuronidase (GUS) and neomycin phosphotransferase II (NPTII) and the "passenger" DNA coding for the antibacterial and antifungal lytic proteins attacin, cecropin, and lysozyme.

Basically, the two transformation procedures (I and II) differed in the (a) duration of the leaf discs' pretreatment period on MS+2% sucrose medium before inoculation (Oh or 48h); (b) duration of *A. tumefaciens* inoculation period of leaf discs (48 or 2); (c) presence of km as selective agent (after or during shoot regeneration). Both procedures of adventitious shoot regeneration were very efficient and shoots started to occur 14 days after cocultivation and peaked 3-4 weeks later; multiple calli and shoots-per-leaf discs were obtained. However, kanamycine and ß-GUS tests on cloned plantlets from randomly-sampled rooted shoots obtained by using procedure I, revealed that none of them had been transformed. On the other hand, 100% of the tested Adventitious shoots, regenerated in the presence of km (transformation procedure II), showed a strong ß-GUS activity.

---

*Dedicated to Prof. G. B. Marini Bettolo on the occasion of his 75th birthday

[1]S. Trinca, C. de Pace, R. Caccia, G. Scarascia Mugnozza. Department of Agrobiology and Agrochemistry, University of Tuscia, Viterbo, Italy.
[2]John H. Dodds. International Potato Center, Apartado 5969, Lima, Peru.
[3]Jesse Jaynes. Department of Biochemistry, Louisiana State University, Louisiana, U.S.A.

Introduction

Although potato (*Solanum tuberosum* L.) is the most important tuber crop in the world (Plucknett, 1984), ranking fourth among all food crops (Horton, 1987), its productivity is impaired by several factors including virus, bacteria, and fungus diseases.

Fungus diseases, compared to virus and bacteria diseases, may be controlled by the use of fungicides. However, the application of fungicides to crops has raised concerns about their environmental compatibility and toxic residue in the edible portion of the plant; in addition, in developing areas, chemical control is hardly feasible for the subsistence farmer.

The use of potato cultivars possessing genetic resistance to fungal diseases is an alternative to chemical control. However, for some fungal pathogens such as *Phytophthora infestans*, which causes late blight, the most serious fungus disease of potatoes, it is difficult to constitute cultivars showing general and effective genetic resistance to the fungus. In potato, 11 Mendelian genes (R genes), the majority transferred from *Solanum demissum*, are known to confer race-specific or vertical resistance to the $2^{11}=2048$ possible races of *P. infestans*, according to a gene-for-gene relationship (Spielman et al., 1989). When these R genes were combined in potato cultivars, they were not sufficient to cope with the high variability of the fungus, and the vertical resistance to *P. infestans* was ineffective. In addition, it is believed that genotypes needing more than one resistance gene, will have to pay a "fitness or metabolic cost" which will probably have a negative effect on yield performance, even in the absence of the pathogen (Burdon and Jarasz, 1990).

What seems more promising is the general resistance (also known as field resistance or horizontal resistance), due to genetic factors that control pathogen infectivity through mechanisms such as cuticle resistance to mechanical forces or enzymes released from pathogens, growth rate of the plants, etc. This type of resistance does not break down rapidly, and has been transferred to *S. tuberosum* from Mexican potato species. It was also found in regenerated plants from culture cells after selection in the presence of the fungal culture filtrate (Behnk, 1980). However, general resistance depends on the specific environment of a potato growing region. Therefore, large breeding efforts are needed (crossing, backcrossing, and recurrent selection) to incorporate general resistance in many potato clones, each specifically adapted to a growing area.

Recombinant DNA technology has opened a broad spectrum of possibilities for improving disease resistance through the isolation and transfer of genes that elicit novel and general mechanisms that may be effective against several plant pathogens in different environmental conditions. Introduction of foreign genes coding for a viral (coat) protein and proteinase inhibitor that confer enhanced resistance to virus and insects to transgenic tobacco plants, respectively, have been reported. Recently, a new class of bactericidal proteins (lysozyme, cecropins, and attacins) have been identified in the pupae of the giant silk moth *Hyalophora cecropia* after a humoral immunity reaction to injected live bacteria (Boman, et al., 1984). These lytic proteins have also shown a potent in vitro antifungal activity against several pathogenic fungi, including *P. infestans*. A cDNA library has been prepared from the mRNA transcribed from the inductive

genes for immunity of *H. cecropia*, and *Agrobacterium* strains harboring selected cDNA sequences corresponding to the attacin and cecropin genes have been constructed.

In this paper we report on the production of regenerated potato plants from leaf discs cocultivated with *A. tumefaciens* constructs, containing attacin and cecropin cDNA and a lysozyme gene from phage fused to the reporter *uidA* (coding for ß-Glucuronidase, GUS) and *npt*II (neomycin phosphotransferase, *npt*II (neomycin phosphotransferase, *nptII*). The ultimate aim of this research is to test the transgenic potato plants for their response to infection with different *P. infestans* races.

Materials and Methods

Plant materials

In vitro-grown plantlets of *Solanum tuberosum* (2n=4x=48) cv.Désirée were used for transformation experiments. The plantlets were propagated in vitro every 3-4 weeks by transferring five single-node stem segments in GA-7 vessels (Magenta) containing 25 ml of MSA solid medium and kept under 16h light (4000 lux)-8h dark photoperiod at 19°C. The MSA medium consisted of the Murashige-Skoog (MS) (Murashige & Skoog, 1962) salts (Flow laboratories) supplemented, before autoclaving, with 2.5% sucrose, 0.1 mg/l gibberellic acid (GA_3), 2 mg/l glycine, 0.5 mg/l nicotinic acid (NA), 0.5 mg/l pyridoxal-HC1, 0.4 mg/l thiamine-HC1, 0.25% phytagel (SIGMA), pH 5.6.

Bacterial Strains

The *A. tumefaciens* strains used were coded A.t. 2 and A.t. 3. They contained a disarmed intermediate vector (pBI121) harboring two reporter genes (*uidA* coding for ß-Glucuronidase and *npt*II coding for neomycin phosphotransferase II), and a passenger DNA sequence coding for attacin (A.t. 1), lysozyme (A.t. 2), and cecropin (A.t. 3) lytic proteins. Each of those genes had its own promoter (Table 1).

Transformation procedure I

Inoculum preparation. The three *A. tumefaciens* strains were prepared for transformation experiments by growing them in liquid LB (5 g/l yeast extract, 10 g/l tryptone, 10 g/l NaCl, pH 7.5) containing 100 mg/l of kanamycin sulfate (km) at 28°-29°C for 36h in a rotary shaker (200 rev/min). The *Agrobacterium* cell density was estimated ($O.D._{600}$), pelleted by centrifugation at 3,500 rpm, and re-suspended in liquid MS supplemented with 2% sucrose, at a final cell density of 10^8/ml.

A. tumefaciens-leaf disc coculture. Leaf discs (1 x 1 cm) were prepared from 3-4 week-old in vitro-grown plantlets, floated upside down on the bacterial suspension for 48h at 26°C in the dark. Then the ex-plants were immersed for 2 min in the sterile distilled water containing 500 mg/l cefoxitina (cfx) (Mefoxin, Merk Sharpe & Dome), blotted dry on Whatman 3MM paper and layered on regeneration CD3Z solid medium containing MS salts supplemented, before

autoclaving, with 2% sucrose, 3mg/l zeatin riboside (ZR), 0.05 mg/l indole-3-acetic acid (IAA), 3 mg/l gibberellic acid (GA_3), 2.5% phytagel, pH 5.6. After sterilization, 500 mg/l cfx was added.

Plant regeneration. The first regenerate shoots appeared 14-15 days from the cocultivation: they were transferred on MSA solid medium without km when 1cm long. Regenerated rooted shoots having 7-8 nodes were separately used for km-resistance test by micropropagating single node stem segments in GA-7 vessels containing MSA solid medium added with km (100 mg/l). The micropropagated plantlets so obtained were tested for GUS activity.

Transformation procedure II

Inoculum preparation. *A. tumefaciens* cultures were prepared in liquid LB as in Procedure I. The cell density was adjusted to 10^8/ml in the same LB medium and 20 ml aliquots were dispensed in 9-cm Petri dishes to be used in the coculture step.

A. tumefaciens-leaf disc coculture. Leaf discs of about 5-6 mm in diameter were excised under aseptic conditions from 3-4 week-old in vitro-grown plantlets. These ex-plants were placed upside down on a circle of sterile Whatman 3MM paper laid on the surface of 25 ml solid medium (MS salts supplemented with 2% sucrose, 2.5% phytagel, pH 5.6) contained in 9-cm Petri dishes, and incubated for two days at 26°C, 16h/day photoperiod, 4,000 lux light intensity (pretreatment). Then the leaf discs were floated for 2 to 3min on a freshly prepared inoculum. After removing excess inoculum by blotting on a sterile filter paper, the leaf discs were incubated, upside down, for two days on the same plates and under the same conditions used for the pretreatment.

Plant regeneration. After the second stage on the pretreatment plates, the leaf ex-plants were transferred to the solid regeneration medium CD3Z containing the antibiotics cfx (500 mg/l), to eliminate bacterial contamination, and km (100 mg/l), to select transformed shoots. The first regenerated shoots appeared after about 14 days on regeneration medium and when they reached 1 cm they removed leaf discs and transferred to solid propagation medium (MSA) added with 100 mg/l km. Rooted plantlets were tested for ß-glucuronidase activity.

ß-Glucuronidase assay

The ß-glucuronidase activity was tested by the fluorometric assay (Jefferson et al., 1987) on leaf discs of the regenerated plantlets in the presence of km from transformation procedure II, and on the micropropagated plantlets from transformation procedure I.

Thirty to 50 mg fresh weight of leaf tissue was ground in extraction buffer (50 mM phosphate buffer pH 7.0, 0.5 mM DTT, 1mM Na_2EDTA, 0.1% sodium lauryl sarcosine, 0.1% Triton-X 100) at a tissue: buffer ratio of 1:3, in a 1.5 ml polypropilene microcentrifuge tube at 4°C with the aid of a glass rod and sea sand. After centrifugation (14,000 rpm, 4') 50 ul of surnatant was reincubated at 37°C in 500 ul of assay buffer which was extraction buffer containing 1 mm 4-methyl umbelliferyl-ß-Dglucuronide (MUG, SIGMA). A different intervals (0', 15', 2h) 100 ul aliquots were removed from the incubated assay buffer and the fluorogenic reaction, if any, was

determined with the addition of 900 ul 0.2 M Na_2CO_3, which also served to develop the fluorescence of the 4-Methyl-umbelliferone (MU, SIGMA) liberated during the fluorogenic reaction. The fluorescence was measured with the Hoefer TKO-100 nM in the 0.2 M Na2CO3 stop buffer. The fluorometer reading was converted into nm of liberated MU per mg of leaf fresh weight.

Results and Discussion

Using both procedures of transformation, callus formation on leaf discs was visible after about 12 days in a regeneration medium, and adventitious shoots started to appear two days later. Callus proliferations were located on the border of the swollen leaf discs near a leaf vein. Multiple calli per leaf disc were formed and some of them produced multiple (up to 50) shoots (Fig. 1). The peak of shoot regeneration was reached within 4-5 weeks for both procedures. The addition of cefoxitina in any medium did not affect shoot regeneration; however, the inclusion of km in regeneration medium (procedure II) caused the production of some bleached shoots. The majority of the calli produced multiple shoots repeatedly over a period of 4-6 weeks even after the leaf discs from which they originated had undergone necrosis (Figs. 2 and 3).

Inoculated leaf discs culture on regeneration medium, without km, using Procedure I, did not show a different pattern of shoot regeneration when compared with leaf ex-plants that had not been inoculated (Table 2). In fact, the proportion of rooted shoots was above 62% in all cases and was 94% when a lower number of leaf ex-plants were used for regeneration. Randomly-sampled rooted shoots were submitted to km test by sectioning each of them into 7-8 single-node stem segments, which were then transferred to MSA medium containing 100 mg/l of km. When more than 50% of the nodes from the same rooted shoot produced a plantlet from the axillary bud, the rooted shoot was considered km-resistant. Using this criterion, more than 80% of the rooted shoots from each treatment, comprising the uninoculated control, were considered km-resistant. Obviously this was an unexpected outcome, and the only explanation was that the rooted shoots regenerated from inoculated leaf discs were not actually transformed, and that 100 mg/l km in the medium was not sufficient to prevent axillary bud growth. In a separate experiment, it was shown that 30% of micropropagated node cuttings of the control shoots in medium containing 150 mg/l of km were still able to produce rooted plantlets.

The ß-GUS assay substantiated the above hypothesis, because none of the sample plantlets, growing in the presence of km produced extracts showing ß-GUS activity (fluorometric reading corresponding to 2-3 nM MU/mg fresh weight. Table 4)

Using Procedure II, callus and adventitious shoot regeneration occurred only on inoculated leaf discs (Table 3). The proportion of shoots that were able to produce roots in a medium containing km ranged from 25 to 64% (Fig. 4). All the randomly-sampled rooted shoots that were assayed for ß-GUS activity produced a very strong fluorogenic signal corresponding to a minimum of 1,538 nm MU per mg of leaf fresh weight. The control shoots originating from uninoculated

leaf discs grown on medium without km, gave very low fluorometric readings corresponding to nm MU/mg fresh weight.

The data show that transformation procedure II, which include km in the regeneration medium, allowed the production of 100% of the regenerated shoots tested that were km-resistant and ß-GUS+. This transformation efficiency was very high also considering that every leaf ex-plant allowed the regeneration of at least one shoot, and that, usually, regenerants from leaf discs show a low somaclonal variation.

In conclusion, it seems that, with Procedure I, only an efficient adventitious shoot regeneration from leaf discs was obtained while, using Procedure II, a high regeneration and transformation efficiency was achieved.

If the above data can be confirmed in repeated experiments Procedure II can be considered the most efficient transformation protocol of leaf discs yet available (Visser et al., 1989; De Block, 1988).

The results of the (a) DNA isolation and analysis from ß-GUS+ regenerated plantlets, and those from (b) cytological observation of root tips, in estimating chromosome number of the regenerated plantlets, will be discussed in another paper.

References

Behnk, 1980. General resistance to late blight of *Solanum tuberosum* plants regenerated from callus-resistant to culture filtrates of *Phytophthora infestans*. Theor. Appl. Genet. 56:151-152.

Boman, H. G., I. Faye, P. Hofsten, K. Kockum, J. Y. Lee, K. G. Xanthopoulos, H. Bennich, A. Engstrom, R. B. Merrified, and D. Andreu. 1985. On the primary structure of lysozyme, cecropins and attacins from *Hyalophora cecropia*. Dev. Com. Imm. 9:551-558.

Burdon, J. J. and A. M. Jarosz. 1990. Disease in mixed cultivars, composites, and natural plant populations: same epidemological and evolutionary consequences. *In* H. D. Brown, M. T. Cligg, A. L. Kohler, and B. S. Wen. Plant population, genetics, breeding, and genetic resources (etc) Sinaner Associates Inc. Suntherlanol, Massachusetts, USA. pp. 215-228

De Block, M. 1988. Genotype-independent leaf discs transformation of potato (*Solanum tuberosum*) using *Agrobacterium tumefaciens*. Theor. Appl. Gent. 76:767-774.

Jefferson, R., T. A. Kawanagh, and M. W. Bevan. 1987. GUS fusion: ß-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J. 6:3901-3907.

Horton, D. 1987. Potatoes. West View Press, Boulder, Colorado, U.S.A. 164 pp.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473-497.

Plucknett, D. L. 1984. Tropical root crops in the Eighties. *In* Proc. Sixth Symp. of the Int. Soc. for Tropical Root Crops. Int. Potato Center (CIP), Lima, Peru. pp 3-8.

Spielman, L. J., B. J. Mc Master, and W. E. Fry. 1989. Dominance and recessiveness at loci for virulence against potato and tomato in *Phytophthora infestans*. Theor. App. Genet. 77:832-838.

Visser, R. G. F., E. Jacobsed, A. Hesseling-Meinders, M. J. Schans, b. Withholt, and W. J. Feenstra. 1989. Transformation of homozygous diploid potato with an *Agrobacterium tumefaciens* binary vector system by adventitious shoot regeneration on leaf and stem segments. Plant Molec. Biol. 12:329-337.

Table 1. *Agrobacterium tumefaciens* strains and their characteristics

Code	*A. tumefaciens* Strain		Intermed. Vector	DNA Sequences	Promoter Expressing: Pass. DNA	NPTII	GUS
A.t.1	LBA	4404	pBI121	Att	Ca2	nos	Ca
A.t.2	LBA	4404	pBI121	Lys	Ca2	nos	Ca
A.t.3	LBA	4404	pBI121	C38	Ca2	nos	Ca

pBI121: pBIN 19 + pCaMV-*b*GUS-Tnos
Att: Attacin
Ca2: double 35S Cauliflower Mosaic Virus promoter; very strong
Ca: 35S Cauliflower Mosaic Virus promoter; strong constitutive
Lys: gene from phage
C38: Cecropin

Table 2. Regeneration efficiency of shoots from potato leaf discs inoculated with various *A. tumefaciens* strains using Procedure I (absence of km in the regeneration medium), and km-test of the obtained rooted shoots.

Bact. Strains	Leaf Discs N°	Regenerat. Efficiency Without km: Shoots N°	Rooted Shoots N°	Rooted Shoots (%)	km-resistance test On rooted shoots: N°	[a] N°	km+ (%)
A.t.1	60	18	17	94.4	5	4	80.0
A.t.2	80	221	150	67.9	30	25	83.3
A.t.3	164	182	113	62.1	29	23	79.3
Control	35	51	48	94.1	15	12	80.0

[a]The rooted shoots were randomly sampled, divided in 7-8 single node, and transferred on MSA + 100 mg/l km medium; the shoots that originated more that 50% of node cuttings with axillary buds growing in presence of km were considered resistant (km+).

Table 3. Regeneration efficiency of shoots from potato leaf discs inoculated with various *A. tumefaciens* strains when Procedure II of regeneration was used.

Bacterial Strains	leaf discs N°	Regeneration Efficiency In presence of 100 mg/l km: Shoots N°	Rooted shoots N°	Rooted shoots (%)
A.t.1	206	186	85	45.7
A.t.2	129	235	150	63.8
A.t.3	264	172	43	25.0
Control	40	0	0	-

[a]The data regarding the regeneration efficiency are incomplete because calli are still producing shoots which will be put on a rooting medium.

Table 4. Fluorometric assay of ß-glucuronidase (B-GUS) activity in randomly sampled adventitious shoots regenerated from leaf discs using transformation procedures I and II.

A. tumefasciens Strain	I Tested shoots	B-GUS act. +	B-GUS act. -	[a]GUS+ (%)	II Tested shoots	B-GUS act. +	B-GUS act. -	[a]GUS+ (%)
A.t.1	16	-	16	-	10	10	-	100
A.t.2	10	-	10	-	16	16	-	100
A.t.3	16	-	16	-	1	1	-	100
Control	10	-	10	-	12	-	12	-

[a]Shoots were considered GUS+ when their lysed tissue released 1538 nM MU/mg of fresh weight.

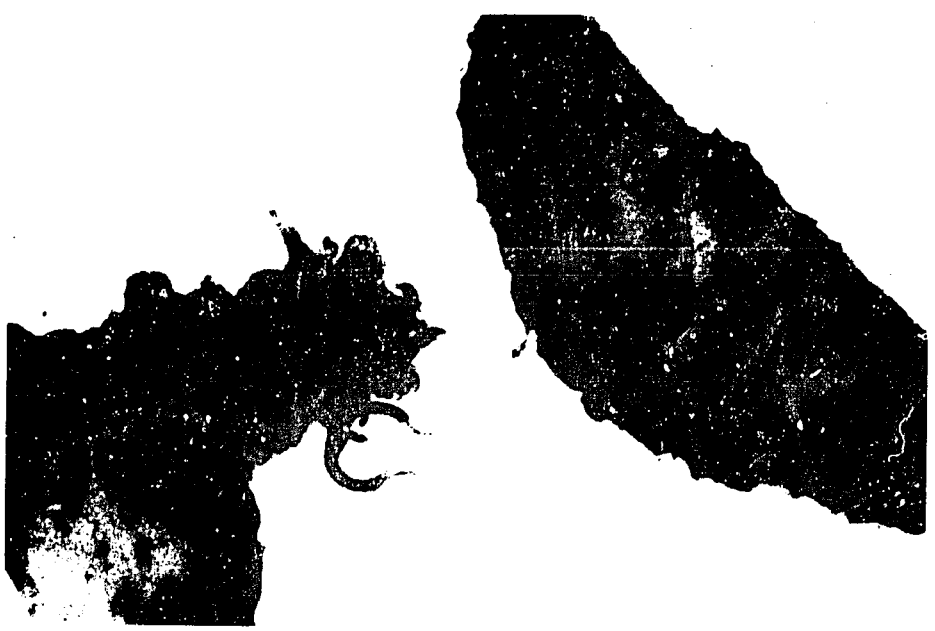


Figure 1. Callus proliferation and multiple shoot formations on regeneration medium + 100 ppm km using potato leaf disc transformation Procedure II (x3).

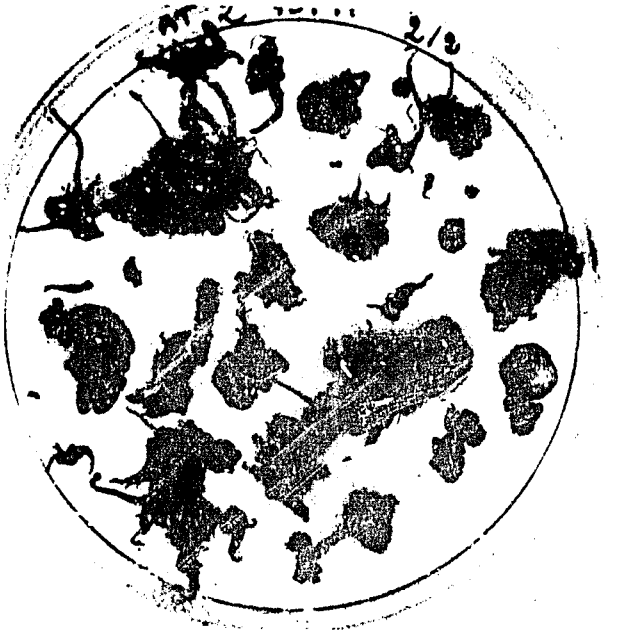


Figure 2. Six-week-old callus and shoot formations on CD3Z regeneration medium + 100 ppm km using potato leaf disc transformation Procedure II and inoculum with strain A.t.2 (x1).

Figure 3. Multiple shoot formations in six-week-old calli on CD3Z regeneration medium + 100 ppm km using potato lef disc transformation Procedure II (x3).

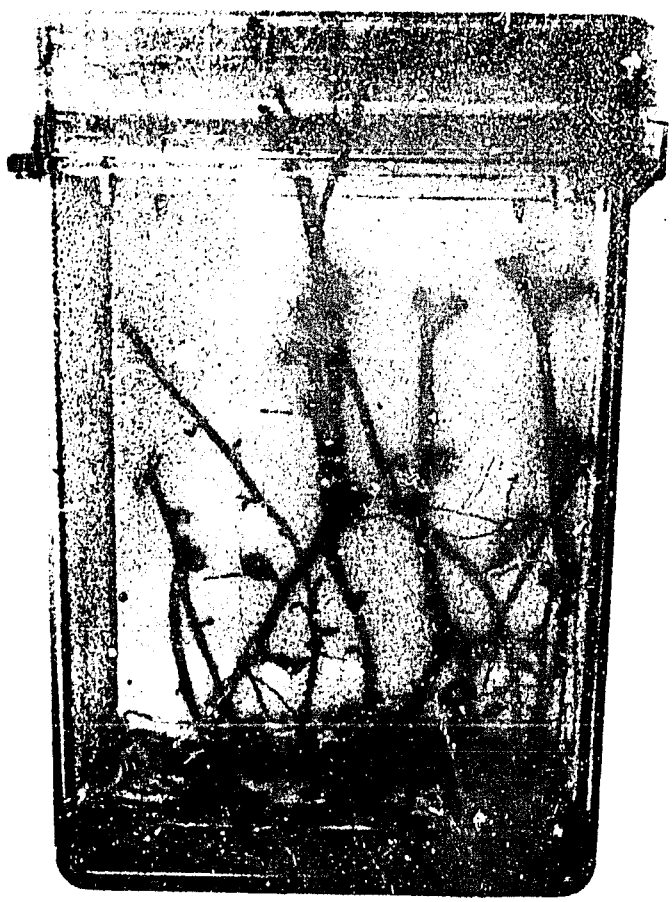


Figure 4. Rooted shoots in MSA + 100 ppm km medium using potato leaf disc transformation Procedure II. (x1.2).

Biotechnology, Biosafety and Field Testing: An Agency for International Development Perspective

J. Cohen[1]

Profile

The Agency for International Development (AID) assists developing country agriculture by: (a) increasing each country's ability to conduct research, (b) improving the delivery of agricultural technology, and (c) contributing to the establishment of an international agricultural research system. It provides this assistance through bilateral projects and core support to the International Agricultural Research Centers (IARCs). In so doing, AID seeks to develop new technologies and products to promote economic and social development in developing countries.

Integrating biotechnology, and specifically genetic engineering, into international agricultural research raises new concerns regarding acquisition and integration of technologies, biosafety, and allocation of scarce research funds. Development strategies are needed to assure the most rational use of finite resources while also ensuring that products of biotechnology can be safely delivered to farmers and consumers. Applications of biotechnology, supported directly by AID, or through the IARCs, are examined in the context of international development, and focus on the need for such research to incorporate appropriate biosafety considerations.

Biosafety and the Developing World

Complications surrounding the establishment of biotechnology regulations in the developing world are due to a variety of influences. Foremost among these is the diverse array of players involved in agricultural biotechnology research and testing (Cohen, et al., 1988). These include the IARCs; donor agencies which provide support for the IARC and national programs; government and research institutions of developing countries; governments, research institutions and private industry of industrialized nations; and various environmental and public advocacy concerns. Each of these groups has its own agenda and its own definition of what constitutes safe and appropriate use of biotechnology products in the environment.

In addition, major constraints to the development of responsible regulatory policy for recombinant DNA research exists in the developing world. Regulatory infrastructure may be non-existent or underdeveloped and unable to cope with the monetary and enforcement

[1]Joel I. Cohen, PhD. Biotechnology Specialist Office of Agriculture Agency for International Development, Washington, D.C., U.S.A.

requirements necessary to establish and implement policy (Umminger, 1989). Establishment of informed public consent, although desirable, may not be now feasible. Therefore, host-country approval will, in most cases, be granted without a national regulatory structure.

Finally, a universal approach to formulating regulatory policy, despite the similarities in concerns and problems facing less developed nations, is non-existent among these countries. Policies among industrialized nations, such as those in Western Europe, also lack uniformity. Nonetheless, the enhancement of host-country capabilities in biosafety, including national abilities to evaluate, approve, and monitor specific requests for testing, is essential if these countries are to fully participate in and benefit from the exchange of this technology with public and private sector elements in more developed countries (Cohen, 1990).

National Research Council Plant Biotechnology for Developing Countries

At the request of the Office of Agriculture, the National Research Council convened a group of experts for discussions concerning priorities in plant biotechnology research that could benefit agriculture in developing countries in the near future (3-5 years). The objective was to identify areas of biotechnology that, in the panel's view, were sufficiently advanced to be ready for promoting in AID-client countries through new collaborative initiatives with U.S. scientific counterparts.

A distinct need was identified for a technical assistance program that encourages and assists developing-country researchers and agencies to acquire the technologies, and focuses on critical problem areas of local importance. A number of priority areas were identified in the meeting, with specific recommendations to be developed upon consultation with sources of expertise most familiar with local problems, needs, and opportunities. The institutional priorities identified included three areas (a) biosafety: AID should assist developing countries to implement and monitor appropriate biosafety regulations; (b) intellectual property: AID should take the lead in developing U.S. policy to promote international cooperation in intellectual property rights, and (c) human resource development and networking: AID should enhance biotechnology capabilities through doctoral and post-doctoral fellowships, and non-degree training for plant biotechnologists.

AID Initiatives

AID support to developing countries establishing responsible biosafety policies for recombinant DNA research would be firmly based in its history of cooperation regarding technical aspects of research and in well-established networks for coordinating activities among national programs, other donor agencies and the IARCs. Other opportunities must be explored to assist the countries no longer eligible for AID assistance.

In the interim, AID and other donors must work cooperatively with each host country to obtain appropriate approval for testing. It has been suggested that assurance should be provided

that research involving genetic engineering, sponsored by the United States government and tested abroad, be conducted under conditions that meet U.S. standards for the protection of health and the environment. Thus, prior to obtaining host country approval, AID (Mellon, 1988) federally-funded research must be in agreement with, and approved by, the appropriate U.S. regulatory agency.

Once approval for domestic testing has been obtained, and the product has been shown to be safe and efficacious in the United States, obtaining approval from a host country should be simplified. However, it is not possible to predict: (1) complications which may arise as national biosafety programs are developed, and, (2) costs required for domestic approval, testing, export, and finally, host-country testing. Further complications will no doubt arise for those products designed for use in the developing world which may not receive the best possible test in the United States.

Transgenic Potatoes

Research in the genetic manipulation of crop plants is being carried out in numerous countries through the CGIAR. The International Potato Center (CIP) has recently begun to apply transformation technologies on potatoes. Transgenic plants have been produced which express amino acids derived from an inserted synthetic gene, referred to as "High Essential Amino Acid Encoding." (Yang, et al., 1988)

This research has involved Louisiana State University (LSU) and CIP in collaborative research supported by AID. Transformed plants have been produced which express a high content of the essential amino acids most deficient among plant-derived proteins. These transformants include improved cultivars from CIP, a local Peruvian cultivar (Mariva) and Russet Burbank. These transgenic plants must remain in greenhouses at LSU and CIP pending regulatory approval for field testing.

A second example could involve cassava or potato genetically engineered for protection against virus infection endemic in Asia or Africa. This will pose difficulties for testing in the United States. However, this can be rectified by testing plants in contained facilities, such as the Foreign Disease-Weed Science Research Unit of the USDA located at Fort Detrick, Maryland. This type of domestic testing, prior to subsequent tests in the developing world, has been carried out in the case of recombinant vaccines, but not yet in the case of transgenic plants.

This is not to say that testing in the United States can substitute for actual host-country testing. Each country has a unique set of environmental conditions, many of these tropical in nature, which cannot be successfully emulated in the United States. For example, concerns have been raised regarding complex crop-weed interactions which may exist between transformed crops and related weed species. In the case of animal vaccines, many diseases are endemic in Africa which do not occur in the U.S. Each of these parameters will have to be carefully considered as confined field testing is initiated. After certain preliminary tests have been

performed and approved through a biosafety review, further confined testing could be initiated at an IARC or host-country national facility (Fig. 1). Data from these tests would lead to final decisions by the developing country itself.

Biosafety Parameters

Public opinion has a direct effect on the processes involved in product development and can be a powerful force in assuring that sound developmental practices are initiated for products of scientific research (Fig. 2). This means providing data to satisfy questions regarding both efficacy and safety for products considered for testing. Public education and awareness should thus be a critical component in the transfer of technology from the laboratory to the field. This is particularly true of new technologies, such as those employing recombinant DNA techniques, since initial public perceptions about product safety and efficacy may have far-reaching implications for further technological advancement.

Thus, maximum coordination will be required to assure that requisite parameters are understood and established for both efficacy and safety. Gaining such information will require direct communication between regulators, principal scientists, and donor agencies. Without establishing such communication links, research projects run the risk of "spinning" the request-review-approval-permit cycle multiple times until appropriate questions have been answered. This will be costly, as each time a request is submitted, costs are entailed for the subsequent testing and documentation.

Once contained testing is warranted, consideration must be given to each host country's ability to monitor the test, its degree of containment, contingency plans if problems occur, termination procedures, and responsibility for supervision. Here also, costs associated with such experimental modifications will need to be estimated within research proposals so that more realistic funding levels can be determined.

While various international organizations, such as the World Health Organization, may assist countries in their approval process, the final decision should be made at the national level. Such approval becomes more complicated in the case of the IARCs, which have a regional or global mandate for their research and which retain special international status, giving them greater autonomy.

In addition, many environmental and safety parameters will vary between those in the United States and each developing country. While some of these may be obvious, special attention will need to be paid to ensure that an appropriate assessment of such differences is obtained well in advance of host-country testing. It would be of great value for regulatory agencies in the industrialized countries to make their personnel available for site inspections and recommendations regarding each proposed test. In the case of AID, such inspections will help ensure that problems are corrected prior to authorizing testing.

However, this necessitates that the domestic regulatory mechanism would have to make itself available for some international travel and provision of recommendations and reports to both agency and host-country biosafety committees. Special training would be required to sensitize domestic regulatory officials to conditions and facilities available in the developing world, putting some additional burden on domestic regulatory agencies but would safely move products of biotechnology to those most in need.

Biosafety and the International Agricultural Research Centers

Few IARCs will be able to develop new basic knowledge in support of biotechnology, rather, they will become involved in the application of new scientific advances. The centers will thus become dependent upon external scientific advances in their effort to adapt technologies to developing-country problems. A strong reliance upon collaborative research exists among centers making advances in biotechnology. Such research efforts between industrialized countries and CGIAR centers will pose new issues once products arise requiring field testing. Both donor countries and the CGIAR center will have to be fully cognizant of biosafety protocol and have in place an approval system which is both rapid and thorough.

Increased activities by the IARCs in adapting the tools of biotechnology to the demands of developing-country agriculture will cause a shift in allocation of resources and personnel. While it will be necessary to continue conventional plant improvement programs essential for integrating new technologies and completing product development, centers may also require personnel to address biosafety concerns, implement biosafety protocols, and meet the novel demands of biotechnology-based research. This alteration in center-based research towards a more strategic direction reflects one of many challenges the IARCs must face regarding advances in technology development.

Insistence by federal agencies to include institutional biosafety review of selected biotechnology-based research indicates the appeal of such an approval process. For example, in addition to the regulatory role of APHIS, the USDA has also established an Agricultural Biotechnology Research Advisory Committee to review research sponsored by USDA. International centers should take action to ensure their research undergoes a similar type of formalized review and is coordinated with host-country approval (Plucknett, D.L. et al., in press) (Fig. 3).

Biosafety Initiatives Within AID

AID established a Standing Committee on Biotechnology in 1987 to serve as an intra-agency mechanism to provide (1) an Agency forum for advice on issues surrounding biotechnology and on the development of mechanisms to address those issues which are technical, regulatory and programmatic in nature, and (2) an entity within AID which will provide liaison with United States Government scientific and regulatory agencies principally concerned with supporting and regulating biotechnology.

The establishment of this committee recognized the role which biotechnology research occupied in AID's development activities and also addressed the Agency's need to safely and responsibly monitor and implement the products of its research. The Standing Committee serves a central purpose in the review and dissemination of information and the coordination of domestic and host-country regulations affecting biotechnology research, and offers specific procedural guidance for AID projects.

The AID Office of Agriculture includes, as a standard provision within grants supporting biotechnology research, specific language regarding regulatory compliance and approval from U.S. regulatory agencies (Jones, 1987). Thus, there is a formal, written requirement by the Office that any risks posed by the research are fully addressed in the context of U.S. government regulations prior to obtaining hostcountry approval. In addition, this approval must be obtained in writing, along with that of the AID in-country mission.

References

Cohen, J. I., K. A. Jones, D. L. Plucknett, and N. J. Smith. 1988. Regulatory concerns affecting developing nations. Bio/Technology 6:744.

Umminger, B. 1989. Biosafety in agricultural research. *In* J. I. Cohen (ed.) Strengthening collaboration in biotechnology: international agricultural research and the private sector. AID, Washington, 437-442 pp.

Cohen, J. I. 1990. International donor support for agricultural biotechnology. Food Policy 15, No. 1: 57-66.

Mellon, M. 1988. Biotechnology and The Environment. A Primer on the Environmental Implications of Genetic Engineering. National Wildlife Federation, Washington D.C.

Yang, M. S., N. O. Espinoza, P. G. Nagpala, J. H. Dodds, F. F. White, K. L. Schnoor, and J. M. Jaynes. 1988. Expression of a synthetic gene for improved protein quality in transformed potato plants. Plant Science, 64:2724-2736.

Plucknett, D. L., J. I. Cohen, and M. Horne. Future role of the IARCs in the application of biotechnology in developing countries. CAB Press, UK. (in press).

Jones, K. A. 1987. AID's activities in biotechnology: regulatory considerations. Contract DAN-1406-0-00-7008-00. AID Washington, D.C.

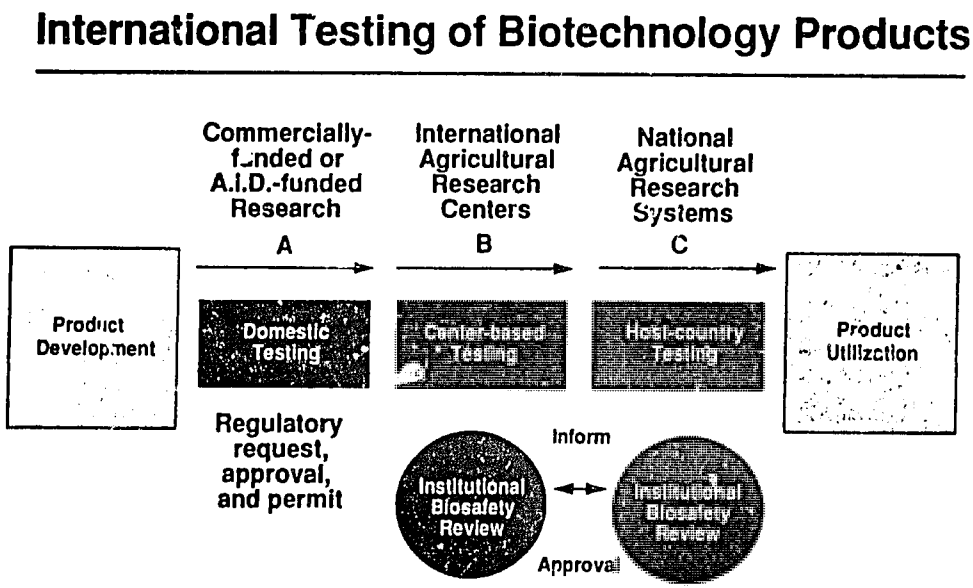


Figure 1. Testing products of biotechnology sponsored by federal agencies involving international centers and eventual host-country testing.

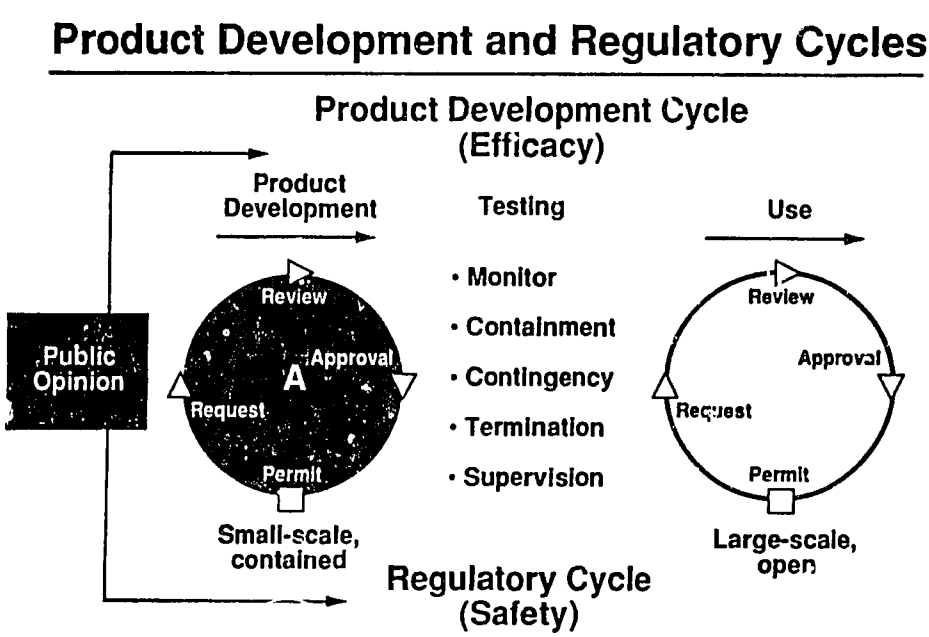


Figure 2. Product development and regulatory cycle with emphasis on efficacy, safety, and approval for testing.

Potential relations between research programs and institutional biosafety committees

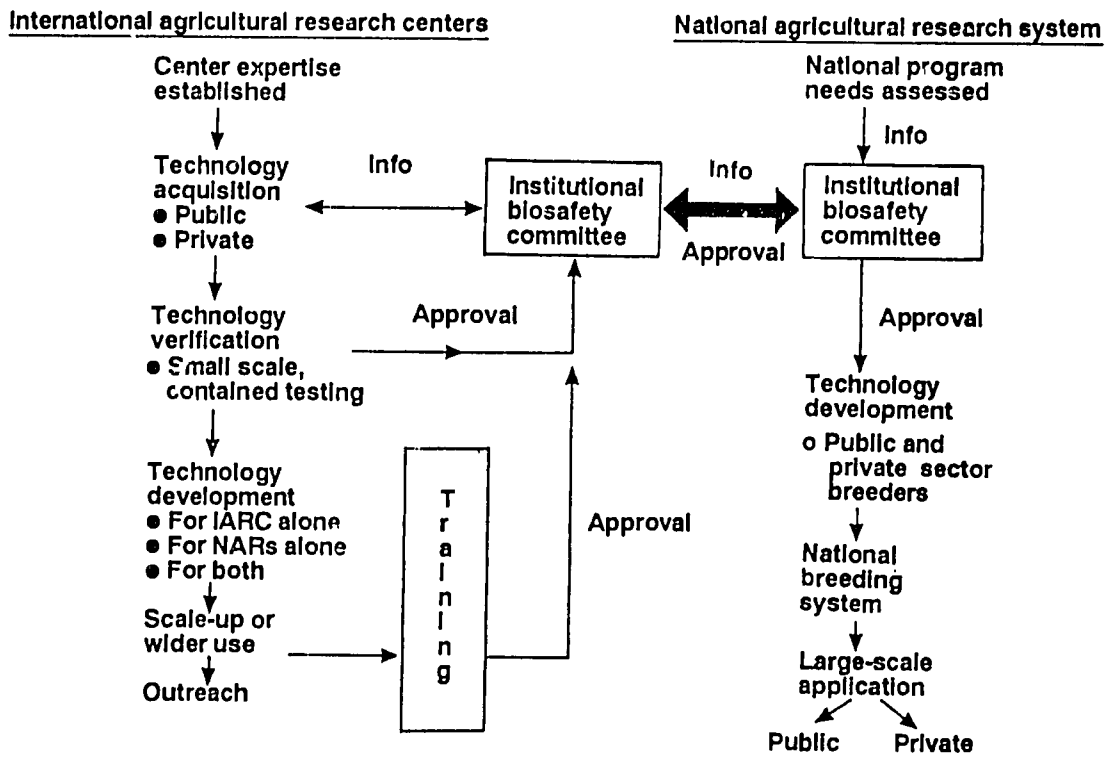


Figure 3. Institutional biosafety committee established at the IARCs in coordination with host-country national programs.

Protoplast Fusion for Potato-Cybrid Formation

E. Galun, D. Aviv, and A. Perl[1]

Profile

Studies in *Nicotiana* and other plant genera indicated that donor-recipient protoplast-fusion is an efficient procedure to transfer chloroplast and traits coded by the mitochondrial genome (chondriome) from a donor species into a target cultivar. We adopted this procedure for *Solanum* as a tool for potato crop improvement and as a means to further investigate interspecific organelle/nuclear-genome interactions. Following biotechnological improvement for the isolation, culture and fusion of potato protoplasts, we developed recombinant DNA techniques to analyze the organelle composition of cybrid plants. Our first cybridizations were designed to render specific potato cultivars into male-sterile seed-parents for the production of hybrid TPS. In further fusion studies we evaluated 14 wild *Solanum* species as donors of plastomes and chondriomes to cybrids having the nuclear genome of the potato cultivar Desiree. Finally, we found that by using a selectable chondriome marker (resistance to oligomycin) the intergeneric barrier between *Nicotiana* and *Solanum* can be bridged. Thus we opened the way to utilizing organelle-coded breeding traits for potato improvement and can convert given potato cultivars into male-sterile female lines for efficient hybrid TPS production.

Introduction

The studies described below were part of an AID-supported project, in collaboration with CIP staff members. Thus the general goals of these studies were formulated with CIP scientists and experts. Our intention was to utilize cell-manipulation methodologies and recombinant DNA techniques, developed in the past in our laboratory for *Nicotiana*, to perform tasks of potato improvement that were of interest at CIP. Since a major task at CIP was the production of TPS and especially of hybrid TPS, our first goal was to convert potential seed-parent cultivars into cytoplasmic male-sterile (CMS) plants. Wild species of *Solanum* are probable sources of organelle-coded breeding traits. Our long-range goal was therefore to evaluate the feasibility of utilizing cybridization for the transfer of either chloroplasts and mitochondria, or both, from these species into potato cultivars. We wondered whether there was a limit for the transfer of these organelles. To answer this question, we choose 14 *Solanum* species as organelle donors and one potato cultivar as a target. We also tested the introduction of a *Nicotiana* chondriome-coded trait

[1]Esra Galun, Dvora Aviv, and Avihai Perl, Department of Plant Genetics, The Weizmann Institute of Science, Rehovot, Israel.

into potato in order to evaluate the transfer of a chondriome component across the sub-family (*Solanaceae*) border.

Experimental Procedures

Our strategy to transfer chloroplasts and chondriome-coded traits from wild *Solanum* species into potato cultivars was based on the donor-recipient protoplast-fusion procedure. This procedure was first described by Zelcer et al. (1978) and was further refined in other laboratories (e.g. Sidorov et al., 1981; Aviv et al., 1986; Galun and Aviv, 1986). It involves the exposure of donor-protoplasts to gamma-irradiation (to arrest further nuclear division) and the exposure of the recipient-protoplasts to an antimetabolite (e.g. iodoacetate, Rhodamine-6-G). Donor and recipient protoplasts are then fused by the addition of polyethylene-glycol followed by dilution with a $Ca(NO_3)_2$ solution. The heterofused protoplasts complement each other and upon culture produce colonies that are regenerated into cybrids having the nuclear genome of the recipient fusion partner but novel organelle compositions. The chloroplasts commonly sort out, causing the cybrids to contain the plastomes of either of the fusion partners. The fate of the mitochondria is different. They rarely sort out as chloroplasts; they usually exchange mtDNA components causing the cybrids to contain novel chondriomes.

To apply the donor-recipient protoplast-fusion procedure to *Solanum*, we had to improve the then-available protoplast-manipulation techniques in this genus. A major improvement was to use silver thisulfate (STS) to increase the yield of protoplasts from in vitro cultured shoots and to render the fusion process more efficient. Ethylene release, during shoot culture in closed boxes, during maceration of leaves by cellulases and pectinases, and during the fusion process, is probably detrimental. STS strongly suppresses these ethylene effects and vastly improves protoplast yields and protoplast fusion (Perl et al., 1988).

For efficient analysis of organelle-composition, we turned to Southern blot hybridization of chloroplast DNA (ctDNA) and of mitochondrial DNA (mtDNA). We thus analyzed the plastome of potato (Heinhorst et al., 1988) and cloned ctDNA fragments that should be useful as molecular probes. Our next step was to find the appropriate restriction endonucleases that would digest ctDNA of potato cultivars and of *Solanum* species into fragments that would be typical for each cultivar and species. Table 1 summarizes our results. Similarly, restriction endonucleases were selected that could differentiate between mtDNAs of potato cultivars and several *Solanum* species (Table 2). We then found that *Nicotiana* mtDNA fragments (Galun et al., 1982) could serve as efficient probes in Southern blot hybridization of *Solanum* mtDNA. The tools for efficient analysis of organelle DNA from *Solanum* cybrids were thus established.

Production and analysis of potato cybrids

During the first phase of our collaborative project, we obtained from CIP axenic shoots of two potato cultivars (Atzimba and Atlantic) as well as shoots of a CMS line (Y245.7) having *S. stolonifenum* cytoplasm. We performed the donor-recipient protoplast-fusion, using protoplasts

of the latter line as organelle donors, and obtained the respective Atzminba and Atlantic cybrids. Several of these were apparently CMS and were sent for further evaluation and integration into CIP breeding systems schemes to A. Golmirzaie.

The second phase of our project was the evaluation of four *Solanum* species (*S. chacoence*, *S. tuberosum*, *S. brevidens*, and *S. berthaultii*) as organelle donors to a potato cultivar (Desiree) recipient. In each case the donor protoplasts were gamma-irradiated (100 Gμ) and fused to iodoacetate (0.25 mM)-treated recipient protoplasts. The fused protoplasts were cultured and the resulting calli were regenerated to obtain shoots that were rooted and transferred to the greenhouse. The morphology, fertility and organelle composition were then evaluated. Figure 1 demonstrates the mtDNA analysis of cybrids resulting from the *S. chacoense* + Desiree and the *S. brevidens* + Desiree combinations. The organelle analyses of the cybrids that resulted from all four fusion combinations are summarized in Table 3. It was found that *S. chacoense* could contribute both its plastome and its chondriome components to the potato cybrids. The latter cybrids were fertile and had normal potato morphology. Plastomes and chondriomes could also be transferred from *S. etuberosum* to potato, but several of the resulting cybrids were male-sterile. Also, pigmentation abnormalities were revealed in cybrids that contained *S. etuberosum* plastomes. When *S. brevidens* was used as organelle donor the regeneration of the fusion-derived calli did not proceed beyond the formation of pale-green shoots. These shoots contained only the donors ctDNA and mtDNA. The combination of *S. berthaultii* donor protoplasts with Desiree-recipient protoplasts did produce fusion-derived calli, but the latter regenerated sluggishly. O-coumarin treatment enabled the regeneration of the respective cybrids, but none of them contained the donors plastome and most of the fusion-derived plants retained the recipients' chondriome. Those that contained the donors' chondriome were male-sterile.

We are currently analyzing 10 additional fusion combinations (Table 4). This study is not yet completed but already indicates that some *Solanum* species (e.g. *S. bukasovii*, *S. venturii*) cannot contribute their organelles into cybrids having *S. tuberosum* nuclear genomes.

Finally, we tested an intergeneric organelle transfer. We used a *N. sylvestris* line (oli$_R$ 38) that harbors oligomycin-resistant mitochondria (Aviv and Galun, 1988) as donor and fused its irradiated protoplasts with iodoacetate-treated Desiree protoplasts. During culture of the fused protoplasts and the subsequent culture and regeneration of the fusion-derived calli, we added gradually increasing levels of oligomycin to the culture media. The regenerated plants had normal Desiree morphology and *S. tuberosum* chloroplasts, but they contained mtDNA fragments (revealed by mtDNA probes with *atp* 6 and *atp* 9 coding sequences) from the oligomycin-resistant *N. sylvestris* donor. Tests with root cultures of a selected cybrid indicated that the oligomycin resistance trait was indeed transferred across the sub-family border.

Prospectives

In the near future, we intend to conclude the analyses of the cybrids resulting from the combinations of all 14 *Solanum* species (as donors of organelles) with potato. We shall then have valuable information on the scope of possible organelle transfer from exotic *Solanum* species into potato. The methodologies developed during our study should also be useful to positively identify *Solanum* accessions.

Our next step will be to utilize amended donor-recipient protoplast-fusion procedures in order to produce asymmetric somatic hybrids. We intend to focus on *Solanum* species that have nuclear-coded breeding traits (e.g. resistances to bacterial and fungal pathogens). Protoplasts of such species will be exposed to strong gamma-irradiation and fused to potato breeding lines. The fusion derived plants will then be exposed to pathogens. It is anticipated that some nuclear genome components will be transferred from the irradiated protoplasts into the potato genome. To render the asymmetric hybridization more efficient, we will introduce selectable markers into the donor plant.

References

Aviv, D., R. Chen, and E. Galun. 1986. Does pretreatment by Rhodamine 6-G affect mitochondrial composition of fusion-derived *Nicotiana* cybrids? Plant Cell Reports 5:227-230.

Aviv, A. and E. Galun. 1988. Transfer of cytoplasmic organelles from an oligomycin resistant *Nicotiana* cell suspension into tobacco protoplasts yielding oligomycin resistant cybrid plants. Mol. Gen. Genet. 215:128-133.

Galun, E. and D. Aviv. 1986. Organelle transfer. Methods in Enzymol 118:595-611.

Heinhorst, S., G. C. Cannon, E. Galun, L. Kenschaft, and A. Weissbach. 1988. Clone bank and physical and genetic map of potato chloroplast DNA. Theor. Appl. Gent. 75:244-251.

Perl, A., D. Aviv, and E. Galun. 1988. Ethylene and in vitro cultures of potato suppression of ethylene vastly improves protoplast yield, plating efficiency and transient expression of an alien gene. Plant Cell Reports 7:403-406.

Sidorov, V.A., L. Menczel, F. Nagy, and P. Maliga. 1981. Chloroplast transfer in *Nicotiana* based on metabolic complementation between irradiated-and iodoacetate-treated protoplasts. Planta 152:341-345.

Zelcer, A., D. Aviv, and E. Galun. 1978. Interspecific transfer of cytoplasmic male sterility by fusion between protoplasts of normal *Nicotiana sylvestris* and X-irradiated protoplasts of male-sterile *N. tabacum*. Z. Pfanzenphysiol. 90:397-407.

Table 1. Potato cultivars and *Solanum* species which were used for the analysis of their plastomes by restriction patterns of the respective ctDNA.

Cultivar or species	Restriction endonucleases used
S. tuberosum cv. Desiree	Bgl I, Bam HI, Eco RI, Xho I, Ava II, PvuII, Hind III
S. tubersoum cv. Atlantic	Pst, Bam HI, Eco RI, Kpn, Hind III, Sma, Ava II
S. tuberosum cv. Atzimba	Hind III, Bam HI, Eco RI, Bgl I, Pvu II, Sma
S. tubersoum	Bam HI, Xba I, Hind III, Pvu II, Eco RI
S. negrum	Bgl I
S. bukasovii	Bam HI
S. stoloniferum	Bam HI
S. acaule	Bam HI
S. chacoense	Pvu II, Sal I, Bam HI
S. brevidens	Bam HI
S. berthaultii	Bam HI
S. commersonii	Bam HI, Hind III, Eco RI, Sal I
S. venturii	Bam HI, Bgl I, Hind III, Eco RI, Sal I
S. demissum	Bam HI, Hind III, Eco RI, Pst I, Pvu II, Sal I
S. microdontum	Bam HI
S. raphanifolium	Bam HI, Bgl I, Hind III, Eco RI, Sal I, Xba I
S. tarijense	Bam HI

Table 2. Potato cultivars and *Solanum* species which were used for the analysis of their chondriomes by restriction patterns of the respective mtDNAs.

Cultivar or species	Restriction endonucleases used
S. tuberosum cv. Desiree	Cla I, Pvu II, Pvu I, Bgl II, Sal I
S. tuberosum cv. Atlantic	Sal I, Bam HI, Bgl II, Eco RV
S. tuberosum cv. Atzimba	Cla I, Bgl II, Pvu I, Pvu II, Sal I
S. tuberosum cv. Kufri jyoti	Bgl II, Pvu I, Pvu II, ClaI, Eco RV, Sal I
S. tuberosum cv. Serrana	Pvu II, Bgl II, Bam HI, Sal I
S. tuberosum cv. 245.7[a]	Sal I, Bam HI, Bgl II, Pvu I, Pvu II
S. brevidense	Eco RV, Cla I, Pvu I, Pvu II, Bgl I
S. chacoense	Cla I, Eco RV, Bfl I, Pvu I, Pvu II, Sac I, Sac II
S. berthaultii	Sal I, Sac II, Sac I, Eco RV, Cla I, Pvu II
S. commersonii	Sac II, Sac I, Eco RV, Cla I
S. venturii	Eco RV
S. acaule	Eco RI
S. demissum	Eco RI
S. etuberosum	Eco RI
S. microdontum	Eco RI
S. raphanifolium	Eco RI
S. tarijense	Eco RI
S. stoloniferum	Eco RI

[a]cv 245.7 contains most probably the citoplasm of *S. stoloniferum*.

Table 3. Organelle composition of protoplast fusion-derived plants (calli) based on Southern blot hybridizations with organelle-specific DNA probes. Protoplasts of the potato cultivar Desiree served as recipients in all fusion combinations.

Donor	Fusion derived Plants (calli)		Organelle analysis				
			Plastome			Chondriom	
	Total	Analyzed	Donor	Recipient	Novel	Donor	Recipient
S. chacoense	38(10)	17 (7)	7	10	1	5	11
S. etuberosum	117(36)	88(33)	31	57	0	13	75
S. brevidens	10 (5)	5 (5)	5	0	0	5	0
S. berthaultii	78(28)	75(22)	0	75	0	8	67

Table 4. Transfer of plastomes and chondriomes (or chondriome components) from *Solanum* species donors to *S. tuberosum* (cv. Desiree). Plastomes and chondriomes were identified in protoplast-fusion derivatives by restriction analysis of the respective organelle DNAs. (updated on December 31, 1989).

Organelle donor species	Organelle DNA identified in cybrids	
	Plastome	Chondriome
S. acaule	+[a]	+
S. bukasovii[b]	*[b]	*
S. commersonii	+	+
S. demissum	+	?
S. microdontum	+	?
S. polyadenium	[c]	?
S. raphanifolium	+	-
S. stoloniferum	+	+
S. tarijense	+	?
S. venturii	*	*

[a]Plus and minus signs indicate transfer or no transfer, respectively.
[b]Asterik indicates that this fusion combination did not yield cybrids, in spite of repeated attempts.
[c]A question mark indicates that the final result will be available within 4 to 6 weeks.

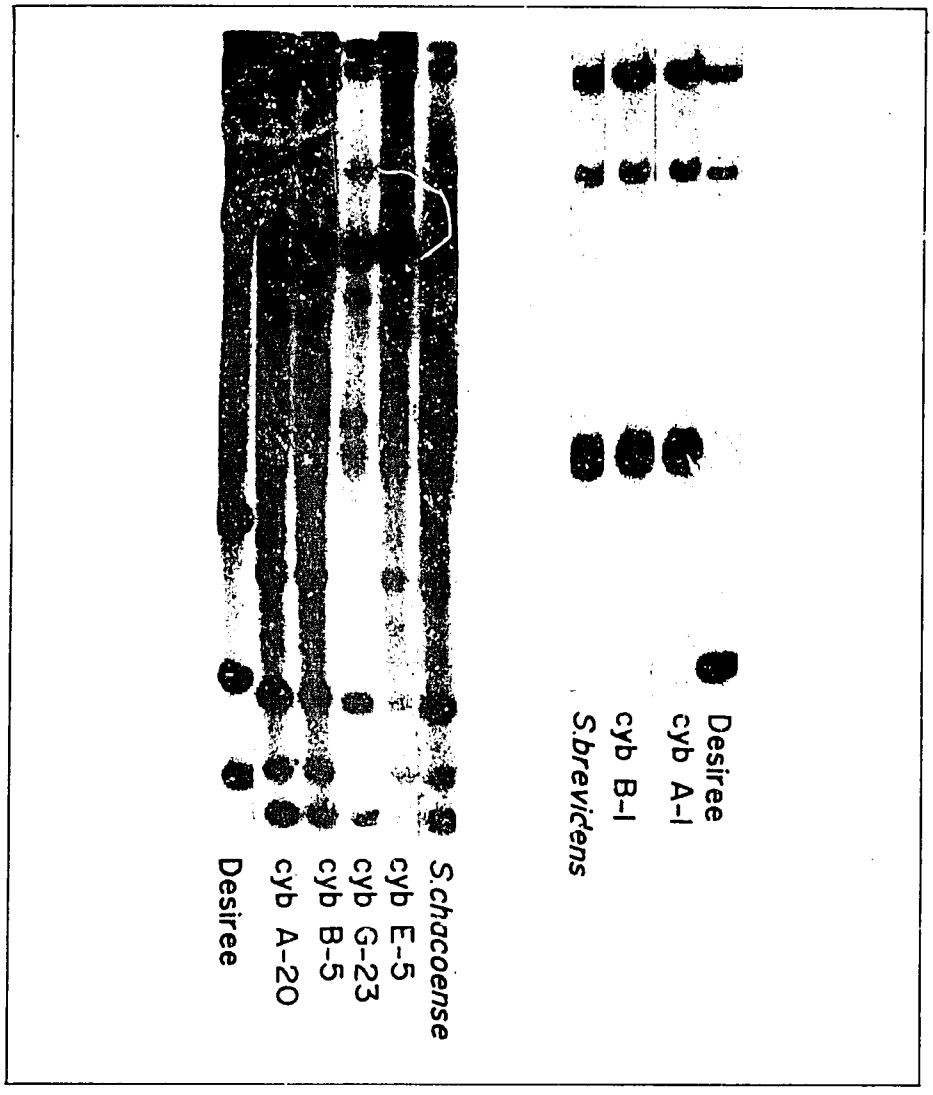


Figure 1. Example to demonstrate the utilization of southern blot hybridization for the characterization of *Solanum* chondriomes. Derivates of two fusion combinations were analyzed and compared to the respective fusion-partners. In the *S. brevidens* (donor) and *S. tuberosum* (recipient) cv. Desiree (recipient) recombination the cybrids contained the donor's chondriomes while in the other combinations (*S. chacoense* with Desiree and others not shown) some cybrids contained 'novel' chondriomes. The DNAs were digested with Hind III. (*S. chacoense*/Desiree) or with Eco RI (*S. brevidens*/Desiree) and Hybridized with radiolabelled *Nicotiana* mtDNA fragments (pmtSy1Sa2 or pmtSy1Sa8).

Use of Protoplast Technology for Genome Transfer.

L. Schilde and H. Ninnemann[1]

Profile

First reported in 1974, the technique of protoplast fusion has entered breeding programs only recently. The delay is caused by the problems encountered when switching from model clones to ordinary breeding lines. In this paper we discuss the important points in the procedure. The introduction of selection markers for hybrid plants from symmetric protoplast fusion in potato is not necessary for potato because hybrid vigor already works in vitro in many cases. The isoelectric focusing of isoenzymes and RFLPs detected with repetitive probes of rDNA or single copy genomic clones allows the unequivocal identification of hybrids.

Asymmetric fusion has been used for the transfer of parts of the genome for several plant species using, mainly, the donor recipient technique. The material incorporated can be selected for the exertion of selection pressure. Selection for complex characters can reveal the incorporation of large genome parts. Careful studies will provide more knowledge of the processes involved in partial genome transfer and might prove the usefulness of this method for breeding purposes.

Introduction

The introduction of single genes or small-sized DNA fragments into a recipient genotype is possible by transformation, using the Ti-plasmid of *Agrobacterium tumefaciens*. Protoplast fusion and related techniques, however, allow the transfer of whole genomes or parts of genomes from a donor to a recipient cell. When somatic hybrid plants were obtained for the first time in 1974 (Melchers and Labib, 1974). There was much optimism about the usefulness of this method for plant breeding. But only recently has the technique entered breeding programs.

One of the reasons for this delay is the difficulty in transferring the method from model clones that respond well in in vitro culture, to ordinary breeding lines. In potato, successful fusions were carried out with at least one partner selected for in vitro culture (Barsby et al., 1984; Helgeson et al., 1988, Debnath and Wenzel, 1987; Deimling et al., 1988) or with a limited number of another culture-derived breeding lines (Karlsson et al., 1988; Waara et al., 1989; Masson et al., 1989).

[1]Lieselotte Schilde-Rentschler and Helga Ninemann Medizinisch, Naturwissenschaftliches Forschungszentrum, University of Tübigen, Germany.

The goal of our project was to develop the technique of protoplast fusion for ordinary breeding lines to a state where it could be used by commercial breeding companies and institutions. This is now happening (Schilde-Rentschler and Ninnemann, 1990).

Conditions for successful protoplast fusion

In our studies we used dihaploid breeding lines from 3 different breeding companies and a governmental breeding program. The clones were selected for resistances and agronomic traits. Working with this material, we found that several prerequisites had to be met for symmetric as well as asymmetric protoplast fusion:

1. *Recovery of viable protoplast*

   a. Preculture of the in vitro plants from which leaflets are used for isolation of protoplasts (photoperiod: 16h; light intensity: 5-7 Jcm^{-2}; temperature 18-20°C; age: 2-3 weeks after transfer, juvenile state).

   b. Incubation in enzyme solutions as brief as possible (two steps).

   c. Culture media V-KM (Binding and Nehls, 1977) and modified MS (Ancora and Tavazza, 1983); embedding of protoplasts in alginate (Schilde-Rentschler et al., 1987); fast reduction of osmolarity of the medium and prompt induction of plant regeneration in medium MS 13 (Behnke, 1978).

2. *Gentle and controllable fusion conditions*

   Fusion of protoplasts, aggregated in a homogeneous alternating electric field by a high voltage pulse, allows control of the size of aggregates and optimization of the fusion frequency of two or three protoplasts maximum (routinely around 15-20%).

3. *Identification of somatic hybrids*

   For symmetric hybrids, preselection by isoelectric focusing (IEF) and testing for esterases were carried out. The selected regenerates were confirmed by RFLPs, using rDNA of 18S-and 25S-rRNA of zucchini as a probe (Schweizer and Hemleben, 1988). For most of the combinations, digestion with one restriction enzyme was sufficient to obtain unequivocal results (Fig. 1, 2 and 3).

4. *Exertion of selection pressure*

   Several strategies have been used for the enrichment of hybrid products in symmetric fusion. For example, the introduction of selectible markers by Ti-plasmid, the selection of deficiency mutants, chlorophyll defects and others. But for applied purposes, selection or induction of mutations as well as introduction of selectible markers are not practical. For potato, it turned out that, for many combinations, somatic hybrids show hybrid vigor even in vitro (Debnath and

Wenzel, 1987; Deimling et al., 1989; Schilde-Rentschler and Ninnemann, 1990). Therefore, the exertion of selection pressure is not necessary for symmetric fusion (Table 1).

Asymmetric protoplast fusion

When a desirable trait under polygenic control has to be introduced into a cultivated line, asymmetric fusion is the method of choice. Also asymmetric fusion could be helpful in obtaining lines with additional chromosomes or chromosome pieces as a basis for chromosome-specific DNA libraries.

Techniques

1. *Inactivation of donor nucleus*

   The method most often used is the donor-recipient technique, originally developed for cybrid formation (Zelcer et al., 1978; Aviv and Galun, 1980), in which the nucleus of the donor protoplast is inactivated by gamma or X-ray irradiation.

2. *Induction of micronuclei (Verhoeven et al., 1988)*

   When synchronized cell suspension cultures are treated with the herbicide Amiprophosmethyl (APM), the regular formation of the spindle apparatus is impaired. Chromosome groups and single chromosomes are dispersed in the cytoplasm. Next, a nuclear membrane is formed around each group. Subprotoplasts containing fragmented nuclei with one or several chromosomes are obtained by centrifugation and can then be fused with the recipient. However, the method is very dependent on the genotype, and more efforts are needed to determine its practical value.

Selection of asymmetric hybrids

Hybrid products can be accumulated through inactivation of the recipient cytoplasm by iodacetate (Nehls, 1978) or Rhodamin 6G (Aviv et al., 1986). Successful transfer of partial genomes has been described for many plant species (Table 2). There are several examples in which the selection was carried out for a nucleus-encoded character of the donor. If asymmetric fusion is to become a useful technique for the transfer of agronomically interesting traits, early identification or selection is necessary. The direct selection for the desired character was possible only in a few cases: in the case of resistance to Phoma-toxin, which acts at the level of cultured cells (Sacristan, 1982; Sjödin and Glimelius, 1989), or of resistance to Alternaria toxin (Behnke, 1979). In model experiments, selection for frost tolerance was successful by transferring this trait from *S. acaule* to tomato (Ninnemann et al., 1987); salt and drought tolerance may be selectible with these techniques (Tal et al., 1978; Van Swaaij et al., 1987).

When intergeneric fusions are carried out, DNA probes of species-specific repetitive DNA interspersed throughout the genome (Ganal et al., 1988) are very useful. This could be proved for

the *Solanum acaule*-tomato hybrids with *S. acaule* and tomato-specific DNA-probes (Schweizer et al., 1988; Röder, 1990).

The data available from asymmetric hybrids show that nuclear hybrid formation is often accompanied by spontaneous chromosome elimination. Minichromosomes were sometimes maintained during mitosis and meiosis, the extent of elimination being higher in more distantly related combinations (Negrutiu, 1989). The chromosomes or pieces of chromosomes of the donor, which are maintained in the asymmetric hybrids, usually possess a centromer. Translocations of chromosome pieces from the donor into recipient chromosomes as well as probable exchange of recipient chromosomes could be demonstrated for *Lycopersicon peruvianum* as donor and *L. esculentum* as recipient (Wijbrandi, 1989). In many cases, the amount of material transferred is larger than desirable. One reason may be the selection pressure exerted, which may specify for extensive parts of the genome (i.e. regeneration capacity in the case of *L. pennellii*). But also in cases in which Ti-plasmid-carried resistance markers were selected, very large pieces of the donor genome have been incorporated (Sacristan et al., 1989). Thus, the regeneration of a plant from a cell colony is a complex process which needs balance of hormone levels and of metabolic processes. Many asymmetric fusion products may not accomplish this balance and therefore do not regenerate to plants. Further studies which carefully try to influence the processes involved in asymmetric fusion (control of cyclecell of donor and recipient, and early selection and identification of products by RFLPs) as well as analysis of large numbers of regenerates, may give us more insight into the processes associated with partial genome transfer. In addition, the efforts carried out for isolation of chromosomes or large DNA pieces of potato (Hemleben, personal communication) may complement the results obtained by asymmetric fusion.

References

Aviv, D., and E. Galun. 1980. Theor. Appl. Genet. 58:121-127.

Aviv, D., R. Chen, and E. Galun. 1986. Plant Cell Rep. 3:227-230.

Barsby, T. L., J. F. Shepard, R. J. Kemble, and R. Wong. 1984. Plant Cell Rep. 3:165-167.

Bates, G. W., C. A. Hasenkampf, C. L. Contolini, and W. C. Fiastuch. 1987. Theor. Appl. Genet. 74:718-726.

Behnke, M. 1979. Theor. Appl. Genet. 54:151-152.

Binding, H., and R. Nehls. 1977. Z.Pflanzenphysiol. 85:279-280.

Debnath, S. C., and G. Wenzel. 1987. Potato Res. 30:371-380.

Deimling, S., J. Zitzlsperger, and G. Wenzel. Plant Breeding 101:181-189.

Dudits, D., O. Fejer, G. Hadlaczky, C. Koncz, G. Lazar, and G. Horvat. 1980. Molec. Gene. Genet. 179:283-288.

Gleba, Y. Y., and F. Hoffmann. 1979. Naturwissenschaften 11:547-554.

Gupta, P. P., M. Gupta, and O. Schieder. 1982. Molec. Gen. Genet. 188:378-383.

Helgeson, J. P., G. Haberlach, J. Pohlman, and S. Austin. 1988. Plant Cell Tissue and Organ Culture 12:185-187.

Karlsson, S. H., H. Tegelström, A. Wallin, and T. Eriksson. 1987. Theor. Appl. Genet. 76:335-341.

Masson, J., D. Laucelin, C. Bellini, M. Lecerf, P. Guercke, and G. Pelletier. 1989. Theor. Appl. Genet. 78:153-159.

Melchers, G. G. Labib. 1974. Molec. Gen. Genet. 135:277-294

Melzer, J. M., and M. A. O'Connel. 1990. Theor. Appl. Genet. 79:193-300.

Negrutiu, I., A. Mouras, Y. Y. Gleba, V. Sidorov, S. Hinnisdaels, Y. Famelaer, and M. Jacobs. 1989. *In* Plant Protoplasts and Genetic Engineering I, Y. P. S. Bajaj (ed). Springer, Berlin.

Nehls, R. 1978. Molec. Gen. Genet. 166:117-118.

Ninnemann, H., M. Bavand, and G. Melchers. 1987. Gesellschaft f. Entwicklungsbiologie, H.D. Pfannestiel (ed). Berlin. p. 27

Röder, M. 1990. PhD-Thesis, University of Tübigen.

Sacristan, M. D. 1982. Theor. Appl. Genet. 75:575-584.

Sacristan, M. D., M. Gerdemann-Knörck, and O. Schieder. 1989. Theor. Appl. Genet. 78:194-200.

Schilde-Rentschler, L., G. Boos. and H. Ninnemann. 1987. *In* Current Plant Science and Biotechnology in Agriculture. K. J. Puite (ed). Kluwer Academic Publ., Dordrecht.

Schilde-Rentschler, L., and H. Ninnemann. 1988. Vortr. Pflanzenzchtg 14:149-163.

Schilde-Rentschler L., and H. Ninnemann. 1990. Vortr. Pflanzenzchtg 14 (in press).

Schweizer, G., and V. Hemleben. 1988. Vortr. Pflanzenzchtg 14:165-173.

Sjödin, C., and K. Glimelius. 1989. Theor. Appl. Genet. 78:513-520.

Sree Ramulu, K., H. A. Verhoeven, and P. Dijkhuis. 1988. Theor. Appl. Genet. 75:575-584.

Tal, M., H. Heikiu, and K. Deham. 1978. Z. Pflanzenphysiol. 86:231-240.

Tavazza, R., and G. Ancora. 1986. Plant Cell Rep. 5:243-246.

Van Swaaij, A. C., J. Nijdam, E. Jacobson, and W. J. Feenstra. 1987. Physiol. Plant. 64:230-236.

Waara, S., H. Tegelström, A. Wallin, and T. Eriksson. 1989. Theor. Appl. Genet. 77:49-56.

Wijbrandi, J. 1989. PhD-Thesis, University of Wageningen.

Zelcer, A., D. Aviv, and E. Galun. 1978. Z. Pfanzenphysiol. 90:397-407.

Table 1. Somatic hybrids produced. Number of analyzed colonies from protoplast fusions, regeneration time, number of hybrids and percentage of abnormal phenotype (most aneuploids or polyploids).

combin.	number col. analysed	regeneration time (months)	number hybrids	% hybrids	% abnormal
R12[a]	390		21	5.4	50.0
R1V2	185	2-4	4	2.2	0.0
R1V1	167	2	0	0.0	
W1821	210	6	4	1.9	25.0
1520[a]	209	4	28	13.4	50.0
0412	28	3	6	21.4	16.7
1523	127	2	3	2.4	0.0
0111	113	2	28	24.8	0.4
0208	13	3	30	25.9	3.2
1516	116	3	30	25.9	3.2
0616	100	3	1	1.0	0.0
0106	113		0	0.0	
1216	137	2	16	11.7	12.5
Nnrs	13	3	9	16.2	11.1
Nsprs	1	6	5	45.5	0.0

[a]Different nurse cells were used for the combinations.

Table 2. Examples of plants regenerated through asymmetric protoplast fusion.

Recipient	donor	selection	proof	authors
Daucus carota chl-	*Petroselinum hortense*	green colonies		Dudits et al., 1980
Arabidopsis. thaliana	*Brassica Campestris*			Gleba and Hoffmann 1980
Nicotiana tabacum	*Daucus carota* MTX^R methyltry^R	MTX^R methyltry^R		Dudits and Pazkowski, 1985
Datura innoxia chl-	*Physalis*	green colonies		Gupta et al., 1984
S. pinnatiseptum	*Solanum phureja chl-*	green colon.	isoenz. anthoc.	Sidorov et al., 1987
Nicotiana tabacum	*Nicotiana plumbaginifolia* Km^R	Km^R	isoenz. Nopalin	Bates et al., 1987
Nicotiana plumbaginifolia NR$^-$, Km^R	*Atropa belladonna*	Km^R, NR$^+$	isoenz. r-DNA	Gleba et al., 1988
Nicotiana plumbaginifolia NR$^-$ Km^R	*Nicotiana tabacum* *Nicotiana sylvestris* *Brassica vulgaris*	NR$^+$, Km^R		Negrutiu et al., 1989
Brassica napus	*Brassica nigra* Hygrm^r	Hygrm^R	isoenz. Nopalin	Sacristan et al., 1989
Brassica napus	*Brassica div species.*	*Phoma*-tox. resistance	RFLPs	Sjödin and Glimelius, 1989
Lycopersicon esculentum Km^r	*Lycopersicon peruvianum*	Km^R, regener. RFLPs	isoenz.	Wijbrandi 1989
Lycopersicon esculentum	*Lycopersicon pennellii*	regeneration	RFLPs	Melzer and O'Connell, 1990

Abbreviations:

MTX	:	Metothrexat
Km	:	Kanamycin
Hygrm	:	Hygromycin
Methyltry	:	Methyltryptophan
R	:	resistance
NR	:	Nitratreductase

Figure 1. Identification of somatic hybrids by RFLPS. For identification an rDNA-probe (pRZ 52) (Schweizer and Hemleben, 1988) and non-radioactive Digoxigenin labeling were utilized. The somatic hybrids B6 (+) B16 as well as B4 (+) B12 are marked by*; B12 (+) B16 by$_{O}$.

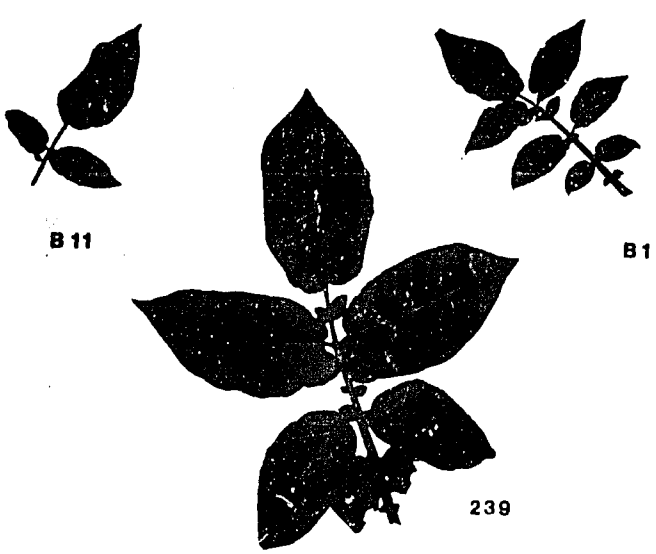


Figure 2. Leaf morphology of somatic hybrids. B1 and B11 are dihaploid breeding lines 239 is one of their somatic hybrids.

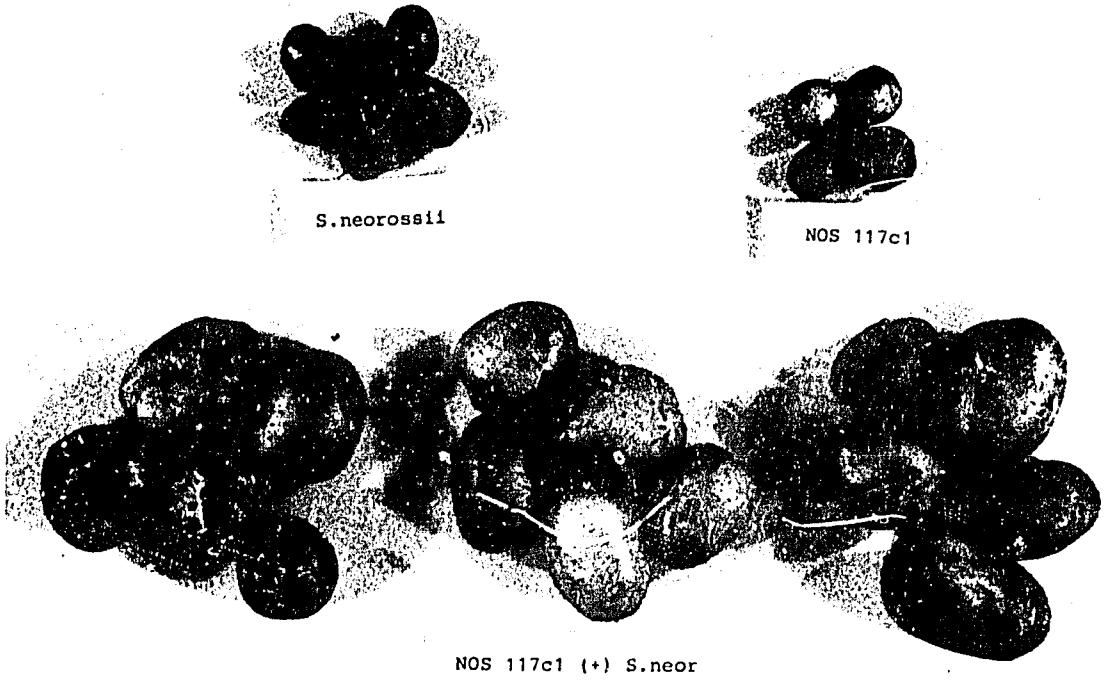


Figure 3. Tuber morphology of somatic hybrids. *Solanum neorossii* protoplast were fused with those of the dihaploid breeding line NOS 117c1. Upper line: the two partners; below: hybrids regenerated from 3 different cell colonies.

Organelle Transfer (mitochondria) for the Production of Cybrid Potatoes and Their Possible Use as TPS Parental Lines

Ali M. Golmirzaie, J. Tenorio and F. Serquen[1]

Introduction

The ultimate goal of research on potato production from true potato seed (TPS) is to develop simple, low cost technologies to achieve a high tuber yield of acceptable quality for farmers in developing countries. Around 50 to 60% of TPS production cost is for emasculation of the female parent. One method of producing low cost TPS is to create a cytoplasmic male sterile (CMS) tetrad type of plant which will attract bees for natural pollination or will allow hand pollination without emasculation. Hybrid seeds can be produced from cybrid plants, like F1 hybrid maize, by interplanting female and male rows of potato in different combinations (number of females/males) and collecting berries just from female rows (cybrids).

Cytoplasmic male sterility (CMS) is one of the characters most widely distributed in the plant kingdom. CMS is a valuable tool in seed production because it is less costly than hand emasculation or nuclear male sterility. Nuclear male sterility makes the work tedious because it is necessary to extract the fertile plants from the field (Hanson and Conde, 1985).

Male sterility can be created in one of the following four ways: a) Intergeneric crossing, b) Interspecific crossing, c) Intraspecific crossing and d) Spontaneous crossing. In the first three cases, the nucleus of a species or cultivar is combined with a foreign cytoplasm which carries organelles from different sources (Edwarson, 1970).

In potatoes, CMS has been found in some provenances of *S. tuberosum, S. phureja, S. maglia, S. vernei, S. chacoense, S. acaule, S. verrucosum* and *S. stenotomun* (Ross, 1986). It is considered as a pre-zygotic crossability barrier caused by a disruptive interaction between certain plasmon types and nuclear genes (Hanneman and Peloquin, 1981).

According to Abdala and Hermsen (1972), who studied the inheritance of the tetrad type sterility in *Solanum verrucosum*, the plants with [tr^r] plasmon will never exhibit tetrad sterility, whereas plants with the sensitive plasmon [Tr^s] will show tetrad sterility only in association with Tr nuclear genes.

[1]Dr. Ali M. Golmirzaie, Jorge Tenorio and Felix Serquen, Breeding and Genetics Department, International Potato Center (CIP), Lima, Peru.

Materials and Methods

Cybrid obtainment

Cybrid production is a joint work between International Potato Center (CIP) and The Weizmann Institute of Science (Israel) with a grant from USAID. The donor, an alloplasmic *S. tuberosum* (*stoloniferum*) line (Y245.7), and recipients, *S. tuberosum* cvs. "Atlantic" and "Atzimba", were sent to Israel. Transfer of cytoplasmic male sterility (mitochondria) to the recipient potatoes (protoplast fusion) took place at the Wiezmann Institute of Science. The fusion was effected by treatment with polyethylene glycol followed by dilution with a solution containing $Ca(NO_3)_2$. The fused protoplasts were then cultured and regenerated. The shoot cultures from this fusion were sent to Lima.

Material evaluation

The shoot cultures received from Israel were first tested (at CIP) to assure lack of pathogens; then they were multiplied and transferred to pots. The plants were sent to Huancayo, one of CIP's experimental stations, where optimal conditions to induce flowering exist. Plants from different calli were studied for various characters to determine if they were true cybrids or not. Plants were evaluated for flower pigmentation (a nuclear coded trait); those with donor flower pigmentation were considered as escapes and were eliminated. The next step was to evaluate pollen from these plants (Table 1).

The methods for determining the phenotypically "good" pollen were as follows:

- Staining with aceto-carmine glycerol. "Good" pollen is round and deeply stained.
- Germination in vitro by the hanging-drop technique using the pollen-medium described by Pallais et al. (1984). The pollen was collected from several flowers immediately after anthesis and it was germinated in this medium.
- In some cases the evaluation of pollen viability was tested using enzymatically induced fluorescence due to the intracellular hydrolysis of fluorescein diacetate in which the pollen grains were immersed (Heslop-Harrison, 1970).

In a second experiment, 41 clones coming from protoplast fusion with low fertility were crossed with clones 7XY.1, LT-7 and Atzimba in order to evaluate the performance of progenies coming from the above population (Table 4) and to examine pollen sterility or fertility (Table 3).

The experiments were conducted at different CIP experimental stations in Peru (La Molina and Huancayo). In a third experiment, clones that had [Tr^S] plasmon (Hermsen 50.3, Y-245.7, C.136 LM86-B and C.662 LM-86B) and one clone coming from protoplast fusion (Gal 1) were used as female parents in order to determine the segregation for tetrad type male sterility in the progeny by means of pollen staining (Table 2). Another objective of this experiment was to study a possible change in the segregation when different sources of tetrad type CMS were used since in Hermsen 50.3 the plasmon [Tr^S] came from *verrucosum* cytoplasm and in Y-245.7, C.136 LM86-B

and C.662 LM86-B, the plasmon came from *stoloniferum* cytoplasm. These progenies were also evaluated for TPS and agronomical characters in a trial conducted at La Molina under a complete block design (Table 5).

In 1989, 600 different genotypes - which included TPS parental lines and pathogen-tested clones planted in Huancayo, San Ramon and La Molina - were evaluated and selected for more CMS types that could be used as donors for protoplast fusion. Six clones with tetrad CMS have been identified out of this population.

Results And Discussion

In 1987, over 45 plants coming from protoplast fusion were evaluated and no true cybrid was found.

In 1988, a total of 14 putative cybrid calli were received. From these calli, 47 plants were regenerated and evaluated for different characters. The evaluation for flower color, in order to determine if there were escapes, showed no plant with the purple flower of the donor. Pollen shape was also evaluated since pollen from plants with tetrad sterility show grains clustered in groups, mostly in tetrads. Germination tests were also performed, and many plants that did not have the tetrad character were fertile but showed very low germination (Table 1).

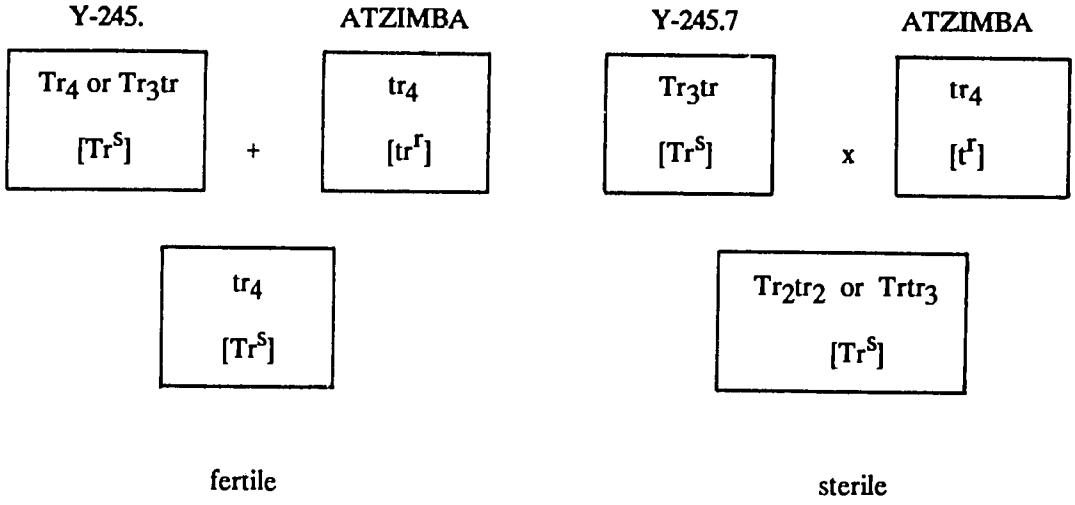
At the beginning of this work, care was taken in the choice of the donor (Y-245.7) but not in that of the recipients. The donor should have tetrad type CMS and the recipient should have nuclear genes that interact with the sterile cytoplasm. In addition, the recipient should have good TPS characters such as medium duration of flowering, profuse flowering intensity, large number of flowers per inflorescence, high fruit set, normal type of anther, bee attraction by flowers, abundant pollen production, and very high seed set as well as good agronomical characters like high yield, round tuber shape, uniform tuber color and plant uniformity for early maturity (less than 90 days).

In the experiment shown in Table 2, several clones possessing different sources of tetrad type sterility (*stoloniferum* and *verrucosum*) including Gal 1 that comes from protoplast fusion (escape), were used as female parents in crossings with several male clones. Irrespective of the source of tetrad CMS, the segregation for this character was 100%. This indicates that the female partners contain the Tr nuclear gene in a triplex or quadruplex state and the male partners have this gene in a nulliplex state because there was no segregation for male sterility. This can be corroborated by Table 3 where clones coming from protoplast fusion (Gal's) were used as females for the same male clones of the previous experiment. The resulting progeny all fertile.

The evaluation of the two groups of plants derived from protoplast fusion has shown that no plant with tetrad type male sterility was obtained. However, these clones (Gal's) have the sensitive plasmon [Tr^s] from the donor and recessive nuclear genes for sterility (nulliplex, tr) from the recipient. The scheme derived is as follows:

$[Tr^S]$ = sensitive cytoplasm

$[tr^r]$ = resistant cytoplasm



Therefore, fertile clones coming from protoplast fusion, can be used as female testers in order to identify male clones with the Tr genes that originate the tetrad type sterility by interacting with the sensitive plasmon. The male clones identified in this way could be used directly in a cybridization program.

After three years of evaluation there are 30 clones with tetrad type CMS - some with *stolonifenum* cytoplasm and others with *verrucosum* cytoplasm - that can be used as donors for protoplast fusion.

New sources of sterility (thirteen clones with tetrad type CMS) will be used to increase the genetic variability of the donors.

Conclusions

Organelle transfer for cybrid potato production for use as TPS parental lines is a work which started only couple of years ago. This period has been mostly a learning stage of the genetics of the tetrad type CMS character.

CMS is a consequence of nuclear mitochondrial genome interactions. Hence the choice of the recipient in protoplast fusion is very important in order to obtain the desired results.

The donor-recipient protoplast fusion system can be used to obtain cybrid plants in potato. The cybrids received from the Weizmann Institute did not show the tetrad type CMS. This character is not phenotypically expressed because the recipient does not have the nuclear Tr genes needed to interact with the [Tr^S] plasmon. This set of clones with sensitive plasmon [Tr^S] may be utilized as female testers in order to identify clones that could be used as recipients in accordance with their nuclear genome.

References

Abdala, M. M. F. and J. G. Th. Hermsen. 1971. The plasmon-genic basis of pollen lobedness and tetrad sterility in *Solanum verrucosum* hybrids and duplicate linkage groups. Genetics 42:261-270.

Abdala, M. M. F. and J. G. Th. Hermsen. 1972. Plasmon and male sterility types in *Solanum verrucosum* and its interspecific hybrid derivate. Euphytica 21:209-220.

Edwarson, J. R. 1970. Cytoplasmic male sterility. Bot. Rev. 36:341 – 420.

Galun, E. and D. Aviv. 1987. Establishment and verification of novel plasmones in higher plants by "donor-recipient" protoplast fusion models in plants. Physiology and Biochemistry. Vol. III.

Hanneman, R. E. Jr. and S. J. Peloquin. 1981. Genetic-Cytoplasmic male sterility in progeny of 4x-2x crosses in cultivated potatoes. Theor. Appl. Gen. 59:53-55.

Hanson, R. M. and M. F. Conde. 1985. Functioning and variation of cytoplasmic genomes: Lessons from cytoplasmic-nuclear interaction affecting male fertility in plants. International Review of Cytology 94:213-265.

Heslop-Harrison, J. and Y. Heslop-Harrison. 1970. Evaluation of pollen viability by enzymatically induced fluorescence, intracellular hydrolysis of fluorescein diacetate. Stain Technology 45(3):115-120.

Pallais, N., N. Fong and D. Berrios. 1984. Research on the physiology of potato sexual seed production. Innovative methods for propagating potatoes XXVIII. Planning Conference. CIP, Lima, Peru. p. 149-168.

Ross, H. 1986. Potato Breeding - Problems and Perspectives. Journal of Plant Breeding Supplement 13.

Table 1. Evaluation of plants derived from protoplast-fusion for color of flowers, pollen shape, and germination.

Fusion-partner	Callus code	Plant No.	Color of flower	Pollen shape	Germination*
Y-245.7 (donor)			purple	tetrad	-
Atzimba (recipient)			white	round	+
	B	1	white	round	+
	8	1,3,13	white	round	-
	8	2	white	round	+
	A	1,4,7	white	round	-
	B	3,4	white	round	-
	B	2	white	round	+
	C	1,2,3	white	round	-
	D	1	white	round	+
	D	2,4,7	white	round	-
	E	2,3,4	white	round	+
	F	1,3,4,5,7,11	white	rouna	-
	F	6	white	round	-
	G	1,2,5	white	round	-
	G	3,10	white	round	+
	I	3	white	round	+
	L	4,5	white	round	+
	M	3	white	round	-
	N	1	white	round	+
	P	1,2,4,6,7,8	white	round	+
	S	1,2,3	white	round	+

* - pollen germination less than 1.5%
+ pollen germination more than 1.5%

Table 2. Determination of tetra type male sterility by stained pollen. La Molina, 1989.

Progeny			Pollen Stainability Observed number Fertile	:	Sterile	Expected Ratios Fertile	:	Sterile
Hermsen 50.3	x	LT-7	0		18	0		1
Hermsen 50.3	x	7XY.1	0		12	0		1
Y-245.7	x	LT-7	0		9	0		1
Y-245.7	x	7XY.1	0		25	0		1
C.662 LM86-B	x	C.342 LM86-B	0		33	0		1
C.136 LM86-B	x	C.614 LM86-B	0		15	0		1
GAL 1	x	7XY.1	0		8	0		1

Table 3. Determination of male sterility. Fourteen families evaluated in Huancayo and La Molina , 1989.a

Progeny		Pollen Stainability Observed number Fertile	:	Sterile	Expected Ratios Fertile	:	Sterile
GAL 6 X	7XY.1	33		0	1		0
GAL 45 X	7XY.1	29		0	1		0
GAL 55 X	7XY.1	48		0	1		0
GAL 84 X	7XY.1	37		0	1		0
GAL 74 X	7XY.1	48		0	1		0
GAL 46 X	LT-7	36		0	1		0
GAL 55 X	LT-7	52		0	1		0
GAL 56 X	LT-7	42		0	1		0
GAL 76 X	LT-7	38		0	1		0
GAL 78 X	LT-7	40		0	1		0
GAL 55 X	ATZIMBA	26		0	1		0
GAL 79 X	ATZIMBA	40		0	1		0

[a]One flower of for each plant was collected and pollen was extracted using a vibrator and stained for examinatiou of male sterility under the microscope.

Table 4. Performance 10 highest families for agronomical and reproductive characters. La Molina and Huancayo, 1989.

Progeny			Yield (t/ha)	No. of berries at harvest	Pollen Production	Flowering intensity	Style lenght	Bees attraction
GAL 52	X	7XY.1	0.930	50.50	2.66	5.66	4.50	1.50
GAL 45	X	7XY.1	0.890	39.50	3.33	5.83	4.00	2.00
GAL 6	X	7XY.1	0.870	457.50	3.66	5.66	4.33	2.00
GAL 71	X	7XY.1	0.860	51.50	3.00	5.33	4.16	1.16
GAL 74	X	7XY.1	0.840	74.67	3.00	6.00	4.66	2.00
GAL 49	X	7XY.1	0.830	42.17	4.00	6.33	4.00	1.50
GAL 79	X	7XY.1	0.820	298.20	3.33	7.33	4.33	2.00
GAL 7	X	7XY.1	0.820	482.20	4.00	6.00	4.50	2.00
GAL 60	X	7XY.1	0.820	69.50	4.00	6.33	5.16	1.50
GAL 55	X	7XY.1	0.810	66.83	4.33	5.66	4.66	1.50
SX			2.63	40.17	0.65	0.60	0.26	0.11
LSD			7.34	112.20	1.18	1.67	0.74	0.32

Table 5. Performance of 8 families for agronomical characters. La Molina, 1989.

Progeny			Yield/plant (Kg.)	Plants at harvest	Earliness
Y-245.7	X	LT-7	0.830 a	19.00	3.66 c
C.136 LM86-B	X	C.342 LM86-B	0.608 ab	25.33 a	4.00 bc
Hermsen 50.3	X	LT-7	0.571 b	19.67 ab	5.00 abc
C.662 LM86-B	X	C.342 LM86-B	0.490 bc	24.33 a	5.33 ab
GAL 1	X	7XY.1	0.479 bc	10.33 c	5.00 abc
Hermsen 50.3	X	7XY.1	0.423 bc	12.67 bc	6.00 a
Y-245.7	X	7XY.1	0.399 bc	16.00 bc	5.66 a
GAL 87	X	LT-7	0.305 c	9.67 c	5.66 a
SX			0.075	2.434	0.477
LSD			0.017	7.382	1.449

Electrophoretic Characterization of Potato Germplasm at CIP

Z. Huamán[1]

The potato collection of native Andean cultivars assembled at the International Potato Center (CIP) in Peru, was originally comprosed of more than 15,000 accessions collected in the potato growing areas in the highlands of Argentina, Bolivia, Chile, Colombia, Ecuador, Guatemala, Mexico, Peru, and Venezuela. Each accession has been maintained by propagation of tubers.

The morphological characterization of the collection showed that there were numerous accessions which were identical to others in all visible characters of the tuber, plant, leaf and flower, i.e. they were duplicates. These duplicates occurred because of repeated sampling of the same cultivar, grown by different farmers in the same geographic region; by sampling widely distributed cultivars several times over extensive geographic areas; and by receiving donations of local, regional or national potato collections.

Data on 18 key morphologic characters were recorded in each accession using a standardized list of descriptors (Huamán et al., 1977). The data were stored in a computerized data management system and multiple comparisons were performed to group those accessions with identical data. Accessions of each group were then grown side by side in the field. Detailed morphological comparisons for all visible plant characters were conducted at flowering time, and at harvest time for tuber characters. Those which were morphologically similar were considered to be potential duplicates.

Since our goal was to reduce the size of the collection by discarding as many duplicate accessions as possible, we wanted to compare other characters that were not related to morphology. By 1975, it was already known that European and North American potato cultivars could be identified by their protein and enzyme banding patterns (Loeschcke and Stegemann, 1966; Desborough and Peloquin, 1968). According to Stegemann (1979), storage proteins are molecules directly coded by the nucleic acid of the genes. The esterases and peroxidases that are extracted from potato tubers are not influenced by environmental conditions or virus infection of the mother plant. A minor change in their patterns can be observed when the tubers are very sprouted. If immature tubers are used, the individual patterns are less pronounced, and one characteristic protein-band is shown.

[1]Zosimo Huamán, Genetic Resources Specialist, International Potato Center (CIP), Lima, Peru.

The presence and position of protein bands from potato tuber extracts are known to be genetically determined (Stegemann, 1975). In fact, Rickeman and Desborough (1978) reported that some protein bands appear to have rather simple inheritance patterns. Thus, they found that one band is inherited by two complementary and independently segregating genes; a second band, by a single dominant gene. A third band was controlled by duplicate dominant genes. Simon and Peloquin (1980) also reported that subsets of eight or four bands were controlled by a single gene, and Mok (1981) added that these proteins were controlled by several alleles of locus Np.

Therefore, CIP decided to use electrophoretic analyses to verify the patterns produced by morphologically similar groups. In theory, all plants of the same clone should produce identical electrophoretic patterns.

Electrophoretic analyses were made at the Institut fur Biochemie, Biologische Bundesanstalt, Braunschweig, West Germany. Proteins and enzymes were used from raw but centrifuged sap extracted from freshly harvested tubers sent from Peru. Five to ten microliters of the sap were separated by standard polyacrilamide (PAA) gel electrophoresis with 6% PAA (ratio acrylamide/methylene-bis-acrylamide 95:5) in tris-borate buffer pH 7.9 or 8.9 for proteins and pH 8.9 for esterases(1 - or 2 - Naphthylacetate as substrata) and for peroxidases (benzidine/H2O2 as substrata). Peroxidases were used less frequently because they are somewhat dependent on the physiological conditions of the tubers. In most cases, the comparison of proteins and alpha-esterases was made in the same gel. Because the majority of the multiple forms of alpha-esterases are found in the lower third (anodic part) of the gel, this portion was cut and stained for alpha-esterases and the rest of the gel was stained with Servablue-R for proteins (Huamán and Stegemann, 1989).

In as much as possible, all accessions considered to be duplicates were compared in the same gel and the cultivar Maritta was always used as a control and also as a spacer. A total of 11,156 electrophoretic comparisons have so far been made in 3,111 groups (Table 1). The analyses made from 1976 to 1983 were conducted in West Germany and the remainder at CIP's facilities in Peru.

In general, the larger the number of identical morphological characters used to determine duplicate accessions, the higher the percentage of identical electrophoretic patterns obtained. Experience has shown that groups made only on the basis of tuber characters have about a 40% chance of identifying duplicates. Many cultivars produce morphologically similar tubers which, when grown, show variation in other characters such as stems, leaves, and flowers.

The use of protein and enzyme patterns alone to identify duplicates in a large number of samples has some practical limitations. Accurate comparisons of similar patterns are very difficult when duplicates are located in several gels. The intensity and position of the bands separated into one dimension cannot be recorded with accuracy using densitometers. The electrophoretic runs are not 100% reproducible, because bands may split slightly or merge, and close neighbors are never resolved (Stegemann, 1983). However, if the cultivars to be electrophoretically compared

are arranged according to their similarities in some morphological characters, the identification of closely related or identical genotypes are significantly facilitated

There have been many instances where slight differences were noticed in the electrophoretic patterns of duplicates. However, in most of these cases repeated analyses in extracts from new tubers showed that the differences were not consistent. In other cases, the electrophoretic results showed different patterns in groups which no doubt were morphologically similar. However, in most of these cases, the differences were due to human error during mixing of either the tuber samples or their extracts.

In general, the electrophoretic patterns of Andean potato cultivars that have been compared through the years at CIP confirmed previous results reported by Rickeman and Desborough (1978b). Thus, some protein bands are common not only to all cultivated potato species but also to wild species. No band was found to be unique to a single cultivated species. However, some bands are more frequent in tetraploid cultivars, but are rarely found in diploid cultivars. Within the same species, the greater the morphological differences, the more distinct their electrophoretic patterns.

In groups of cultivars that are closely related, the number of identical bands was very high. However, they differed slightly because one or few bands were be absent or did not have the same intensity. If the difference was at least one band, the cultivars also showed some morphological differences.

It has also been possible to document cases in which closely related cultivars of the same species showing differences in tuber skin, stem or flower color produced identical electrophoretic patterns of proteins and alpha-esterases.

However, these could be differentiated by patterns of beta-esterases (Huamán and Stegemann, 1989). Thus, in the pentaploid species *S. x curtilobum*, there are 3 cultivars producing white, purple, and two-colored tubers, as well as some minor differences in the distribution of anthocyanin pigmentation throughout the plant. The same has been noticed in some cultivars of the other hybridogenic-cultivated species, the triploids *S.x juzepczukii* and *S.x chaucha*, and the diploid *S. x ajanhuiri*. A possible explanation for this is that the phenotypic variation in these cultivars might be due to somatic mutations whose effects in the genetic architecture of the plant are limited to genes affecting color expression and therefore, do not greatly affect their electrophoretic patterns.

If there are considerable genetic changes in closely related cultivars, they are also clearly differentiated by their banding patterns. This variation has also been observed by Smith (1986), who reported that somaclones generated from protoplasts of some potato cultivars showed differences in the concentration of a protein band, ranging from complete absence to a heavily staining wide band. The electrophoretic variation was attributed to some fundamental alteration in the genetic material or in its expression, since the plants also showed morphological and

physiological differences. Although doubling the chromosome number does not produce noticeable differences in protein and esterase patterns (Desborough and Peloquin, 1967), the plants do show differences in size, vigor, etc.

Electrophoretic differences have also been shown even when the morphological differences are minor. Thus, for example, a variant of clone DTO-33 that produced tubers with a different tuber eye color showed an identical but blurred protein pattern when analyzed by standard PAGE. However, it could be differentiated from the original clone by the different concentration of some bands using two-dimensional electrophoresis after focusing in a narrow pH range (Huamán and Stegemann, 1989). This finding shows that these techniques could also be applied in studies of genetic stability of materials stored in in vitro culture. In that case, it would be important to detect any change in the genotypes of the materials conserved in this form.

Both morphological and electrophoretic analyses have been shown to be effective tools for the identification of potato cultivars. Biochemical analysis is extremely useful to differentiate potato cultivars that show very minor morphological differences. The application of morphological and electrophoretic comparisons for identification of duplicants in the Andean potato cultivars maintained at CIP has helped reduce the collection from more than 15,000 accessions to about 5,000, representing approximately 3,500 different genotypes (Huamán, 1986). Photographs of their electrophoretic patterns are kept together with the original tuber extracts. These materials are to be used to check for genetic changes in plants of the same cultivar regenerated from in vitro stocks.

References

Desborough, S. and S. J. Peloquin. 1967. Esterase isozymes from *Solanum* tubers. Phytochemestry 6:989-994.

Desborough, S. and S. J. Peloquin. 1968. Potato variety identification by use of electrophoretic patterns of tuber proteins and enzymes. Amer. Potato J. 45(6):220-229.

Huamán, Z. 1986. Conservation of potato genetic resources at CIP. CIP Circular 14(2):1-7.

Huamán, Z. and H. Stegemann. 1989. Use of electrophoretic analyses to verify morphologically identical clones in a potato collection. Plant Varieties and Seeds 2:155-161.

Huamán, Z., J. T. Williams, W. Salhuana, and L. Vincent. 1977. Descriptors for the cultivated potato and for the maintenance and distribution of germplasm collections. Rome, IBPGR. 47 p.

Loeschcke, V. and H. Stegemann. 1966. Proteine der kartoffel-knollen in abhangigkeit von sorte und virosen (Polyacrylamid-elektrophorese). Phytochemistry 5:985-991.

Mok, I. G. 1981. Sexual polyploidization and protein diversity in potatoes. Ph.D. Thesis, University of Wisconsin-Madison.

Rickeman, V. S. and S. L. Desborough. 1978a. Inheritance of three electrophoretically determined protein bands in potato (*Solanum tuberosum* L.) Theor. Appl. Genet. 52:187-190.

Rickeman, V. S. and S. L. Desborough. 1978b. Elucidation of the evolution and taxonomy of cultivated potatoes with electrophoresis. Theor. Appl. Genet. 52:217-220.

Simon, P. W. and S. J. Peloquin. 1980. Inheritance of electrophoretic variants of tuber proteins in *Solanum tuberosum* haploids. Biochem. Genet. 18:1055-1063.

Smith, D. B. 1986. Variation in the electrophoretic band pattern of tuber proteins from somaclones of potato. J. Agric. Sci., Camb. 106:427-428.

Stegemann, H. 1975. Properties of and physiological changes in storage proteins. *In* Harborne, J. B. and C. F. van Sumere (eds.). The Chemistry and Biochemistry of Plant Proteins. New York, Academic Press. pp. 71-88.

Stegemann, H. 1979. Characterization of proteins from potatoes, and the Index of European Varieties'. *In* J. G. Hawkes, R. N. Lester and A. D. Skelding (eds.). The Biology and Taxonomy of the *Solanaceae*. London, Academic Press. pp. 279-284

Stegemann, H. 1983. Retrospect on 25 years of cultivar identification by protein patterns and prospects for the future. *In*: Proc. Int. Seed Testing Assn. on Biochemical Tests for Cultivar Identification. Cambridge, Cambridge University Press. pp. 20-31.

Table 1. Number of cultivated accessions from CIP's collection that were compared in their protein and enzyme patterns.

Year included	Electrophoretic series	Number of groups compared	Number of accessions
1976	1,000	74	489
	2,000	94	964
1977	3,000	123	495
	4,000	144	969
1978	5,000	229	1,026
	6,000	340	1,035
1979	7,000	346	908
1980	8,000	411	638
1981	9,000	429	1,622
1982	10,000	447	1,512
1983	11,000	312	1,031
1986	12,000	162	467
		3,111	11,156

Present Status of Germplasm Enhancement at CIP and Application of Biotechnology

K. Watanabe[1]

Profile

Advancements in germplasm enhancement at CIP are discussed, especially in relation to use of wild species at the diploid level. Also discussed are problems with introgression of wild germplasm into the cultivated-potato gene pool.

Future work in biotechnology to overcome the present obstacles is presented.

Genetic resources for germplasm enhancement

Objectives of the germplasm enhancement program at CIP are:

1) to broaden genetic diversity in the CIP collection;

2) to introduce the genetic resistances/tolerances against biotic and abiotic stresses into the cultivated potato gene pool. Exotic tetraploid potato cultivars collected throughout the world, from primitive cultivated species such as *S. phureja*, *S. goniocalyx*, and *S. stenotomum*, and some of their related wild species have been used as sources of resistances and for widening genetic diversity (Table 1).

It should be pointed out that many sources of resistances/tolerances originated from limited groups within the tuber-bearing *Solanum*, which contains over 200 species, including numerous accessions.

Germplasm enhancement through the use of diploids and 2n gametes

Germplasm enhancement at the diploid level is a unique way of capturing many valuable genes from diploid wild species for use in the diploid cultivated gene pool, and to efficiently select and improve these hybrids. Disomic inheritance at the diploid level is significantly more efficient than tetrasomic inheritance at the tetraploid level, which requires much larger population size for selection. The main scheme of the germplasm enhancement employed at CIP is shown in Figure 1.

[1]Kazuo Watanabe, Genetic Resources Dept., International Potato Center, Lima, Peru

Haploids induced from advanced tetraploid breeding lines are the main source for capturing valuable genes from wild species and maintaining cultivated characters in hybrids with wild species. Some progress has been made in inducing elite haploids with some genetic attributes (CIP, 1987). These haploids can also be utilized to establish genetic bases of resistances in tetraploid potatoes.

2n gametes, especially 2n pollen generally occur in potatoes and their related wild species (Watanabe and Peloquin, 1989). 2n pollen has been employed as a tool to tranfer genetic attributes from the improved diploid hybrids to tetraploid potatoes. 2n pollen is usually formed by parallel spindle and it is genetically equivalent to first-division restitution. The mechanism of 2n pollen formation is controlled by a single recessive gene (Mok and Peloquin, 1975). 2n pollen (2x) from diploids transmits about 80% of parental heterozygosity and genetic compositions in contrast to about 60% of parental genetic constitution in normal pollen (2x) from tetraploid (Peloquin, 1983). Some quantitatively inherited traits such as resistances to root-knot nematode and bacterial wilt are transmitted to tetraploid via 2n pollen (Iwanaga et al. 1989, unpublished data).

One advantage of germplasm enhancement at the diploid level is that combining resistances in one genotype is much easier than in tetraploids (Peloquin, 1983). Some progress has been made in combining resistances in diploid genotypes with 2n pollen and further attempts are being made to accumulate the resistances in particular genotypes (CIP, 1988).

Utilization of disomic tetraploid species as new sources

Resistances/tolerances to Potato Virus X, Potato Virus Y, frost and late blight seen in the tetraploid breeding lines and cultivars, originated from disomic polyploid wild species in many cases (Ross 1986). Disomic tetraploid species *Solanum acaule* and *S. stoloniferum* have high levels of resistances/tolerances compared with those of cultivated tetraploid potatoes, and these are sometimes unique types of resistance. (CIP, 1989, Hanneman and Bamberg, 1986; Ross, 1986). Great efforts were made to incorporate the resistances/tolerances from disomic tetraploid species (Ross, 1986). However, it took decades to attain and incorporate the genetic attributes. The delay was mainly due to 1) cross incompatibility caused by endosperm breakdown (Johnston et al., 1980) and 2) linkage drug (Ross, 1986).

An innovative approach has been made at CIP to overcome the cross-incompatibility problems between tetraploid potatoes and disomic tetraploid wild species. A combination of counterfeit pollinations and embryo rescue was employed to obtain hybrids. Tetraploid hybrids were obtained by this method (Iwanaga et al., 1991), and some progress was made in introgressing wild genes into the cultivated-potato gene pool. It appears that this method provides more probability of enhancing recombination between cultivated potato genomes and disomic tetraploid genomes than other methods, thus resulting in less linkage drug. An important aspect of this method is that the tetraploid hybrids can maintain workable levels of both male and female fertility and for intergenomic recombination (Iwanaga et al., 1991).

Obstacles in germplasm enhancement

Some progress has been made in germplasm enhancement; however, there are several large obstacles to proceed more efficiently. Some of these are listed below:

1. Appropriate genetic markers are lacking;
2. Many target characters of interest are quantitative inheritance;
3. (Host genotypes x pathogens) x environments interactions occur; thus, identifying and utilizing resistances is very complicated;
4. Identification of a particular potato chromosome is difficult, thus, manipulation of individual chromosomes is extremely difficult.

Overall, these problems can be attributed to the difficulty in monitoring introgression of target genes and in the elimination of undesirable characters, especially from wild species. Furthermore, confirmation of transmission of resistance(s) from progenitors by progeny-testing is time-consuming but essential, since several genetic factors such as cytoplasmic effects and female-by-male interactions influence the expression of resistance in progenies.

Identification, definition, screening methods, and mechanism of resistance/tolerance are also important components to establishing a basis for accelerating germplasm enhancement. The sources of resistances should be surveyed widely since currrent sources are limited and the level of resistance is not high enough to achieve potato improvement.

Planning for overcoming the obstacles

The recent development of restriction fragment length polymorphisms (RFLPs) is one major area which CIP should emphasize to overcome the present obstacles in germplasm enhancement (Tanksley et al., 1989). With further development of RFLP technology on diploid potatoes by Bonierbale et al. (1988), the monitoring in introgression would be facilitated in the future. The main targets of RFLP work in this area will be:

1. Linkage analysis by RFLP to detect DNA markers which co-segregate with target genetic characters;
2. RFLP-assisted screening and selection; and
3. RFLP map-based cloning of target-resistant genes.

The genetic resources should be exploited to identify new sources of resistances, and hopefully, higher levels of resistances.

There are three major groups of species which should be exploited (Hanneman and Bamberg, 1986; Ross, 1986):

1) Diploid species indexed by 2 EBN should be surveyed more since these species can be manipulated easily;

2) Further development of utilization scheme for disomic tetraploid species will help to incorporate strong and unique resistances in *S. acaule* and *S. stoloniferum*; and

3) Diploid species indexed by 1 EBN are also of interest; species such as *S. brevidens* have very high levels of resistances to potato leaf roll virus and *Erwinia* spp., however, these species cannot be sexually hybridized with potatoes by conventional methods. Application of protoplast fusion technology is essential at the moment, and this should be developed further to facilitate the effective use of these species.

References

Bonierbale, M., R. L. Plaisted, and S. D. Tanksley. 1988. RFLP maps based on a common set of clones reveal modes of chromosomal evolution in potato and tomato. Genetics 120:1095-1103.

CIP 1987. Annual report, International Potato Center, Apartado 5969, Lima, Peru.

CIP 1988. Annual report, International Potato Center, Apartado 5969, Lima, Peru.

CIP 1989. Annual Report, International Potato Center, Apartado 5969, Lima, Peru.

Chavez, R., C. R. Brown, and M. Iwanaga. 1988. Application of interspecific sesquiploidy to introgression of PLRV resistance from non-tuber-bearing *Solanum tuberosum* to cultivated potato germplasm. Theor. Appl. Genet. 76:497-500.

Hanneman, R. E., Jr. and J. B. Bamberg. 1986. Inventory of tuber-bearing *Solanum* species. Bulletin 533, College of Agriculture and Life Sciences, University of Wisconsin, Madison, Wisconsin, USA.

Iwanaga, M., P. Jatala, R. Ortiz, and E. Guevara. 1989. Use of FDR 2n pollen to transfer resistance to root-knot nematodes into cultivated 4x potatoes. J. Amer. Soc. Hort. Sci. 114:1008-1013.

Iwanaga, M., R. Freyre, and K. Watanabe. 1991. Breaking the crossability barriers between disomic tetraploid *Solanum acaule* and tetrasomic tetraploid *S. tuberosum*. Euphytica In press.

Johnston, S. A., T. P. M., den Nijs, S. J. Peloquin, and R. E. Hanneman, Jr. 1980. The significance of genic balance to endosperm development in interspecific crosses. Theor. Appl. Genet. 57:7-9.

Mok, D. W. S., and S. J. Peloquin. 1975. The inheritance of three mechanisms of diplandroid (2n pollen) formation in diploid potatoes. Heredity 35:295-302.

Peloquin, S. J. 1983. Genetic engineering with meiotic mutants. *In* Mulcahy, D. L. and Ottaviano, E. (eds.), Pollen: Biology and Implications for Plant Breeding. Elsevier, New York, pp.151-155.

Ross, H. 1986. Potato breeding - Problems and Perspectives. Verlag Paul Parey, Berlin.

Tanksley, S. D., N. D. Young, A. H. Paterson, and M. W. Bonierbale. 1989. RFLP mapping in plant breeding: New tools for an old science. Biotech. 7:257-260.

Watanabe, K. and S. J. Peloquin, 1989. Occurrence of 2n pollen and *ps* gene frequencies in cultivated groups and their related wild species in tuber-bearing *Solanum*. Theor. Appl. Genet. 78:329-336.

Table 1. Genetic background and sources of resistances/tolerances used in potato improvment.

Trait	Genetic background/source[a]
Late blight	ADG, TBR(DMS, STO)
Early blight	PHU, STN
Cyst nematode	ADG, BUK, CHC, PHU, SPL, STN, TBR
Root-knot nematode	ADG, CHC, MCD, PHU, SPL, STN, TBR
Bacterial wilt	ADG, CHC, MCD, PHU, SPL, STN, TBR
Erwinia spp.	PHU, STN
Potato tuber moth	ADG, PHU, SPL, STN, TBR
Heat	CHC, PHU, STN, TBR
Potato viruses X and Y	ADG, TBR(ACL, STO)
Potato leaf roll virus	ADG, TBR

[a]Abbreviation based on Simmonds (1963).

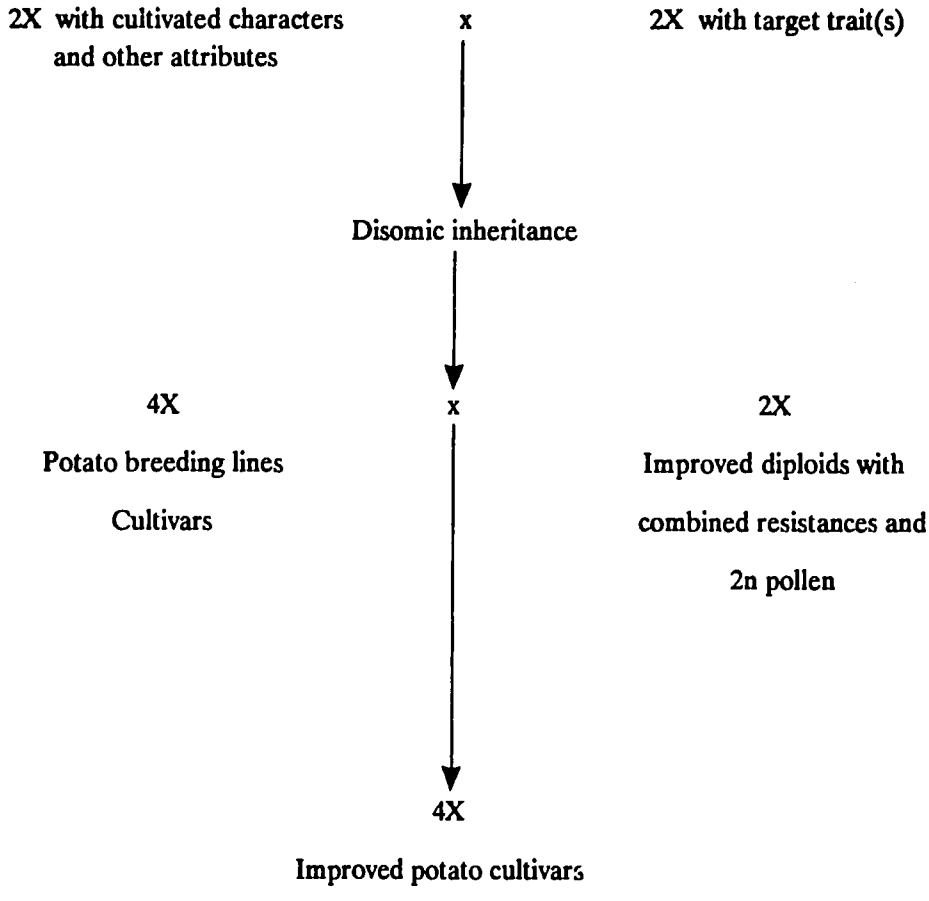


Figure 1. Central scheme of germplasm enhancement at diploid level and transfer of genetic attributes from diploids to tetraploid potatoes by 2n pollen.

Induced Variation for Potato Improvement

A. Sonnino[1]

Introduction

By exploiting the largely unexplored reservoir of the genus *Solanum*, to which more than 1,000 species belong, it is possible to transfer a number of valuable genes into the cultivated potato. Several improved cultivars now on the market have been obtained by interspecific cross and further significant improvement can be realistically expected in this field.

Nevertheless, the introduction and spread of improved potato varieties is often limited by the market. Consumers are often accustomed to the cooking quality and taste of traditional cultivars and therefore prefer them to the new varieties (Howard, 1978). "Well established" varieties are cultivated throughout the years in some third world countries, because of the "rusticity" and good adaptation, and potato growers resist the introduction of outyielding cultivars (Brown, 1983). Some of these old varieties lack important traits such as pest, disease, and stress resistance, cycle length, tuber appearance, dormancy. The high amenability of potato in cell and tissue culture permits the application of methods of somatic cell genetics to this species. This paper will briefly review the research work done at ENEA's Casaccia Center for the development of valuable methodologies of in vitro induction of mutation and for the exploitation of somaclonal variation. The possibilities offered by these technologies for in vitro selection of disease-resistant clones will be also discussed.

In Vitro Mutation Breeding

In potato advanced vegetative generations by micropropagation is achieved in a matter of months, instead of the years needed with in vivo propagation. The apical dome of a bud belonging to an in vitro-grown shoot is generally formed by fewer cells than the apical dome of buds of in vivo plants (Decourtye, 1983; Locardi, 1984). The buds of micronodes consist of a limited number of fast multiplicating and genetically stable cells, representing therefore a very convenient target for mutagenic treatments. Micropropagation, in fact, reduces diplontic selection and chimera formation, enhances the frequency of mutation, and facilitates the recovery of solid mutants (Ancora et al., 1983; Donini and Micke, 1984).

[1]Andrea Sonino, ENEA-Agrobiotechnology Department, C. R. E. Casaccia, Rome A. D., Italy

Rapid isolation of solid mutants after micropropagation of mutagenized buds has been reported for a number of species, including Weigelia (Duron and Decourtye, 1986), cherry rootstocks (Ancora et al., 1982) and Musa (Novak et al., 1987).

At ENEA we irradiated a dose of 30 Gy (gamma-rays) in vitro-growing shoots of potato (cv. Desirée) (Sonnino et al., 1986). Single-node segments were cut from the irradiated shoots and subcultured on fresh medium. The plantlets obtained from the subcultured buds have been cut in single-node segments and subcultured a second time. The plantlets obtained from the second cycle of micropropagation were taken out of the test tubes and transplanted in the open field (Figure 1).

Among the 1,094 plants analyzed, we recognized 102 mutants, bearing 158 mutuations for several morphological characters. More than one third of the mutants detected (34.3%) were chimera or gross aberrants. The remaining mutants, evaluated throughout several vegetative generations, proved to be stable and homogenous. Some of them appeared to be promising from an agronomic point of view. In particular, one mutant, with white-skinned tubers, seems to retain the good agronomic value, adaptation and tuber quality of the mother variety. Considering the better acceptability for the Italian potato market of white potatoes, the mutant has been named "Desital" and submitted to official trials for registration on the National Variety List (Sonnino and Ancora, 1988). "Desital" has been approved and is now being multiplied and released to the farmers by an Italian commercial firm. In vitro mutation breeding is now applied to improve other outstanding varieties (Sonnino et al., 1988b). For instance, a mutant of the variety "Cruza-148" lacking the red vascular ring characteristic of the mother variety, has been obtained and is now being evaluated at CIP. If the resistance to bacterial wilt and the good agronomic aptitude of "Cruza 148" are retained by the mutant, it has the potential for overcoming the acceptance obstacles of the starting variety.

Exploiting of Variation Generated by Culture

The possibility of exploitation of somatic variation generated from culture of protoplasts and of somatic tissues was evaluated in a specific experiment (Sonnino et al., 1987, 1990a and 1990b). Plantlets have been regenerated from mesophyll protoplasts following the technique of Tavazza and Ancora (1986) and from explant of somatic tissue (stem, petiole, leaf) according to the procedure described by Sonnino et al., (1988a). Protoclones and somaclones have been field evaluated throughout three vegetative propagation cycles.

The highest amount of morphological variability (33.62%) has been observed among the protoclones. Unfortunately, 42.8% of the variants were gross aberrants and 15.5% chimeras and therefore of no use for breeding purposes (Figure 2). The regeneration from leaf, stem and petiole explants induced 24.3% of morphological variants, 10.8% of which were aberrants and 18.9% chimeras.

The higher rate of gross aberration in protoclones than in tissue-derived plants can possibly be explained by the high amount (more than 50%) of aneuploides or other chromosome abnormalities reported in protoplast-derived plants (Sree Ramulu, 1987).

These results suggest that the regeneration of adventitious buds from somatic tissues, besides being less laborious, is a more suitable method for induction of variation in potato than protoplast regeneration.

Tissue culture-derived variation can also be used to duplicate the chromosome number of diploid clones (Sonnino et al., 1988a).

In Vitro Selection for Disease Resistance

Both in vitro mutation induction and somaclonal variation could have more advantageous applications when simple, reproducible, and reliable methods of in vitro selection are available. The application of field selection procedures on in vitro-induced variants entails the risk of reducing the advantages gained by in vitro induction of variation, and is highly laborious and time-consuming. On the contrary, in vitro selection allows an easy handling of large populations under controlled and reproducible conditions.

The insensitivity of plant tissue or cell cultures to culture filtrates of pathogenous fungi has been shown to correlate fairly well to plant resistance to a number of diseases (Buiatti, 1987).

In our laboratory, we studied the parameters needed to screen in vitro mutagenized shoots for insensitivity to culture filtrate of *Phytophthora infestans* (Crino et al., 1988). A culture on liquid medium containing fresh potato extract and dextrose (2%) for 21 days allowed a fast growth of fungus mycelium, gave a culture filtrate with a toxic effect on cultured potato nodes higher than the culture filtrates obtained with other media, and interfered only slightly with potato shoot growth when added to micropropagation medium. It must be emphasized that the medium Henniger (1959) normally utilized to grow the fungus mycelium (Behnke, 1979) has an inhibitory effect on potato nodes, and can therefore interfere with the selection process.

The culture filtrate added to the agarized potato micropropagation medium showed toxic effects only on the shoot tissues directly in contact with the medium (stem bases, roots), thus appearing inadequate for performing selections. A liquid selective medium, composed of the organic and inorganic constituents of the micropropagation medium diluted with 50% of culture filtrate, was later adopted (Crino et al., 1989), and the selection scheme described in (Figure 3) was followed.

Of 1,200 axillary buds developed from 240 irradiated buds, 95 (7.9%) survived a first cycle of screening in liquid selective medium and 65 (5.6%) overcame a second cycle of challenge (Penuela et al., 1989). The plants derived from insensitive buds have been inoculated in vitro with a fungus sporangia suspension and 8.5% of them had no or only slight symptoms (0-2 score on a 0-5 scale). Further tests are in progress to clarify the response of these plants to the disease.

Conclusion and Perspectives

Tissue culture seems a convenient system to induce genetic variation in potato. The induced variation includes detrimental as well as potentially useful changes and the relative quantity of deleterious and advantageous variation is highly dependent on the regeneration process. Since breeders often seek slight changes in outstanding varieties, regeneration from somatic tissue explants appears able to induce more suitable variation, minimizing the induction of drastic, undesired changes.

The micropropagation of mutagenized shoots seems promising as well because of its ease and rapidity and of the possibility of fine tuning the variation induced by changing the irradiation dose.

The variation induced by in vitro culture includes resistance to a number of diseases (Matern et al., 1978; Shepard et al., 1980; Jellis et al., 1984; Thomson et al., 1986; Evans et al., 1986).

The application of selective pressures directly in vitro during the culture can greatly enhance the efficiency of induction of disease resistance. Our preliminary results of in vitro selection of mutagenized buds for insensitivity to culture filtrate of *Phytophthora infestans* seem promising. The genetic nature of the observed insensitivity to culture filtrate must be clarified. The possible application of this technique depends on the existence of correlation between culture filtrate insensitivity and increased late blight tolerance.

Selection procedures at the cellular level make it possible to handle very large populations of potential individuals, increasing the probability of recovery of useful mutants. On the other hand, a population of cells growing in vitro is far different from a whole plant growing in a field, while the selection performed on plantlets (Meulemans et al., 1986) or shoots (Crino et al., 1988) is much closer to field conditions, but it is feasible only on a limited number of individuals. The most suitable selective agent to be used (culture filtrate, purified toxins, chemical compounds, etc.) also needs more investigation.

The potential impact of in vitro selection justifies, in our opinion, the great deal of research effort needed, even though it must be emphasized that only the field test can give the final proof of an induced pathogen resistance.

References

Ancora, G., E. Benvenuto, L. Cuozzo, B. Donini, and G. Roselli. 1982. Micropropagation of cherry rootstock F 12/1 clones originated from irradiation: the isolation of solid mutants. Riv Ortoflorofrutt It 66: 231-238.

Ancora, G., E. Benvenuto, A. Sonnino, and L. Cuozzo. 1983. Colture in vitro e mutagenesi delle piante a propagazione vegetativa. *In*: Atti della Giornata di Studio sull'uso di tecniche nucleari per il miglioramento genetico dei fruttiferi. Rome, ENEA, Casaccia: 41-61.

Behnke, M. Selection of potato callus for resistance to culture filtrates of *Phytophthora infestans* and regeneration of resistant plants. Theor Appl. Genet 55: 69-71.

Buiatti, M. 1987. Nuovi metodi di miglioramento genetico per la resistenza a stress biotici. Agricoltura e ricerca 77: 33-42.

Brown, C. R. 1983. Potato breeding though mutagenesis in aposporously generated true seed clones of established varieties. *In* Research for The Potato in The Year 2000. International Potato Center, Lima, Perú. pp. 67-68.

Crino, P., R. Penuela, L. Martino, A. Sonnino, and G. Ancora. 1988. Reaction of in vitro potato micronodes to culture filtrate of *Phytophthora infestans*. *In* A. Graniti et al., Phytotoxins and Plant Pathogenesis, NATO ASI Series, Vol. H27, Springer Verlag, Berlin Heidelberg, pp. 441-442.

Decourtye, L. 1983. Bilancio di 20 anni di miglioramento delle specie legnose da frutto mediante la mutagenesi e prospettive attuali. *In* Atti della Giornata di studio sull'uso di tecniche nucleari per il miglioramento genetico dei fruttiferi. Rome, ENEA, Casaccia, 21-40.

Donini, B. and A. Micke. 1984. Use of induced mutations in improvement of vegetatively propagated crops. *In* Induced mutations for Crop Improvement in Latin America. IAEA Tech. Doc. 305: 79-98.

Duron, M. and L. Decourtye. 1986. Effects biologiques des rayons gamma appliqués á des plantes de Weigela cv. (Bristol Ruby) cultivées *in vitro*. Int. Symp. Nuclear techniques and *in vitro* culture for plant improvement. IAEA, Vienna, pp. 103-111.

Evans, N. E., D. Foulger, L. Farrer, and S. W. J. Bright. 1986. Somoclonal variation in explant-derived potato clones over three tuber generations. Euphytica 35: 353-361.

Jellis, G. J., R. E. Gunn, and R. E. Boulton, 1984. Variation in disease resistance among potato somaclones. Abstr. Conf. Pap. Trienn. Conf. EAPR pp. 301-381.

Henniger, H., 1959. Versuche zur Kultur veschiedener Rassen von *Phytophthora infestans* (Mont.) de Bary auf kunstlichen Nahrboden. Phytopathologische Zeitschrift 34: 285-306.

Howard, H. W., 1978. The production of new varieties. *In* P. M. Harris (ed.). The potato crop, the scientific basis for improvement, Chapman and Hall, London, pp. 607-646.

Locardi, C. 1984. Induzione e isolamento di mutanti in *Solanum tuberosum* mediante tecniche di coltura in vitro. Tesis di Laurea, Univ. La Sapienza, Rome.

Matern, U., G. Strobel, and J. F. Shepard. 1978. Reaction to phytotoxins in a potato population derived from mesophyll protoplasts. Proc Natl Acad Sci U.S.A. 75: 4935-4939.

Meulemans, M., D. Duchene, P. Tegera, and G. Fourage. 1986. Selection of variants by dual cultures on potato and *Phytophthora infestans*. *In* J. Semal (ed.) Somaclonal variations and crop improvement. Martinus Nijhoff Pbl, Dordrecht, pp. 219-223.

Novak, F. J., B. Donini, T. Hermelin, and A. Micke. 1987. Potential for banana and plantain improvement through in vitro mutation breeding. Memorias VII Reunion ARCOBAT, San José, Costa Rica, Boletín Técnico No. 121, pp. 67-70.

Penuela, R., L. Martino, A. Lai, PO. Crino, A. Sonnino, and G. Ancora. 1989. Selezione in vitro per resistenza a *Phytophthora infestans* in patata. Atti del XXXIII Convegno Nazionale, Societa Italiana di Genetica Agraria, p. 45.

Shepard, J., D. Bidney, and E. Shanin. 1980. Potato protoplasts in crop improvement. Science 208: 17-24.

Sonnino, A., and G. Ancora. 1988. Desital: A new potato variety developed by in vitro mutation breeding. Mutation Breeding Newsletter 31:3.

Sonnino, A., G. Ancora, and C. Locardi. 1986. In vitro mutation breeding of potato: the use of propagation by microcuttings. *In*: Int Symp Nuclear techniques and in vitro culture for plant improvement. IAES, Vienna, pp. 385-394.

Sonnino, A., M. Iwanaga, and A. Henestroza. 1988. Chromosome number doubling of 2x potato lines with diverse genetic background through tissue culture. Potato Research 31: 627-631.

Sonnino A., V. Papacchioli, V. Beinat, and G. Ancora. 1988b. Development of improved potato varieties by means of in vitro mutation breeding. Genetica Agraria 42 (1): 94.

Sonnino, A., R. Tavazza, V. Beinat, and G. Ancora. 1987. Variability in protoplast-derived plants as compared to variability induced by in vitro irradiation of shoots. Abstr. Conf. Pap. Trienn. Conf. EAPR, pp. 184-185.

Sonnino, A., R. Tavazza, P. Crinó, R. Penuela, L. Martino, and G. Ancora. 1990a. Induction and use of variation in potato with different in vitro systems. Acta Horticolturae (in press).

Sonnino, A., R. Penuela, P. Crino, L. Martino, and G. Ancora. 1990b. In vitro approaches for induction of genetic variability and for selection of disease resistant plants in potato R. Lester, N. Estrada, M. Nee, J. G. Hawkes (eds.). *Solanaceae*: Chemistry, Evolution, Biotechnology (in press).

Sree, R K. 1987. Genetic instability during plant regeneration in potato: origin and implication. Life Sci Adv (Plant Physio.) 6: 211-218.

Tavazza, R. and G. Ancora. 1986. Plant regeneration from mesophyll protoplasts in commercial potato cultivars (Primura, Kennebec, Spunta, Desirée). Plant Cell Reports 5: 243:246.

Thomson, A. J., R. E. Gunn, G. J. Jellis, R. E. Boulton, and C. N. D. Lacey. 1986. The evaluation of potato somaclones. *In* J. Semal (ed). Somaclonal variations and crop improvement. Martinus Nijhoff Pbl., Dordrecht. pp. 236-243.

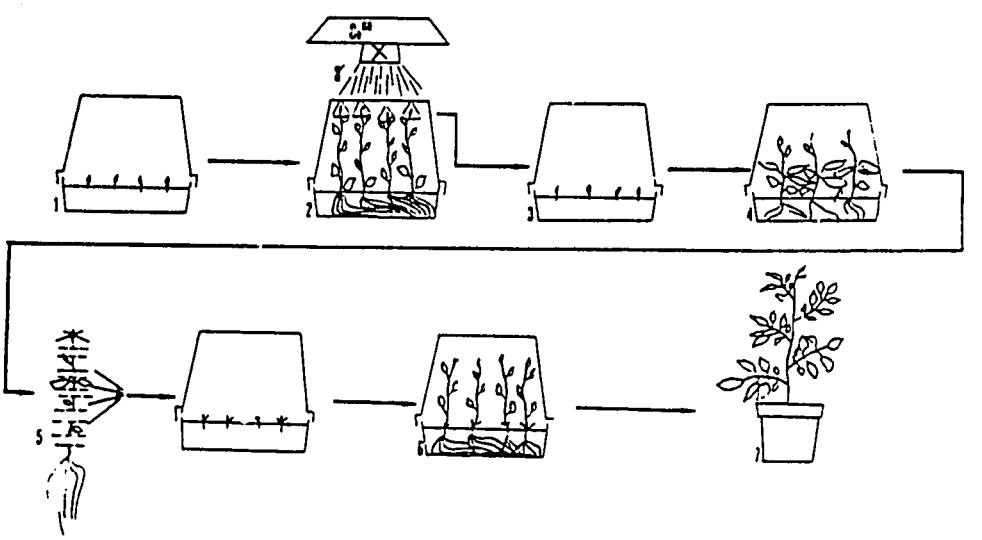


Figure 1. Chart of procedure for induction of variation by irradiation and micropropagation of shoots.

1. Potato micronodes cultured *in vitro*. 2. Irradiation of plantlets derived from micronodes. 3. Culture on fresh medium of apical buds taken from irradiated plantlets. 4. Growth of plantlets resulting from apical buds. 5. Excision of 4-5 axillary nodes and their culture on fresh medium. 6. Growth of shoots from excised nodes. 7. Adult plant transferred into pot.

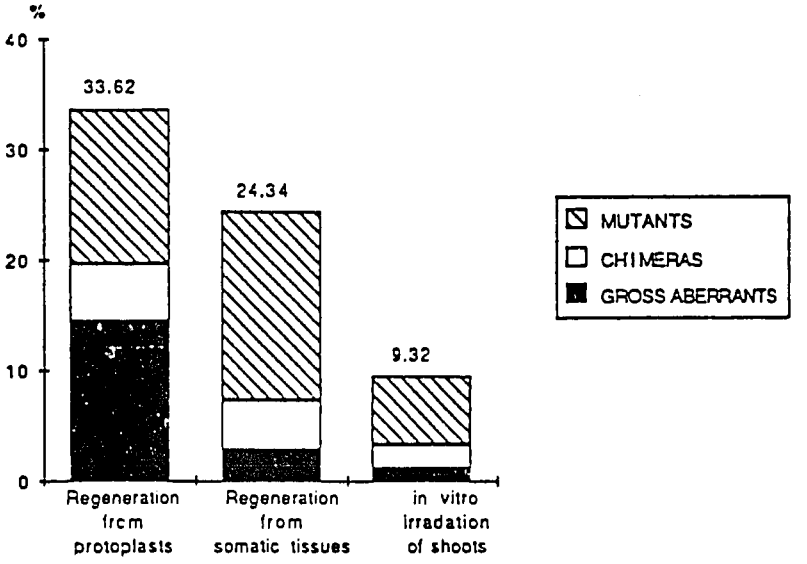


Figure 2. Kind and extent of variation induced by different in vitro systems in potato.

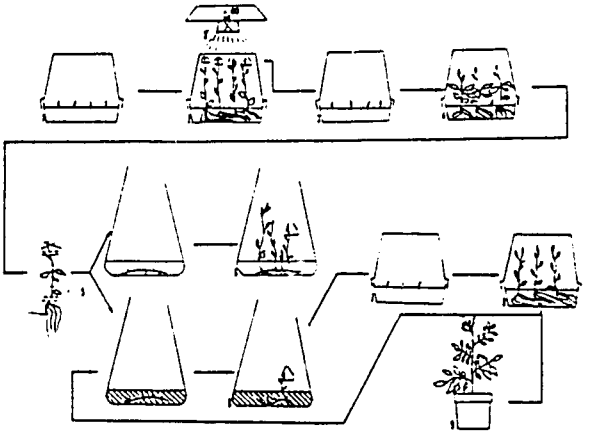


Figure 3. Chart of procedure for *in vitro* irradiation of potato shoots and selection for insensitivity to culture filtrate of pathogenic fungi.

1. Potato micronodes cultured *in vitro*. 2. Irradiation of plantlets derived from micronodes. 3. Culture on fresh medium of apical buds taken from irradiated plantlets. 4. Growth of plantlets resulting from apical buds. 5. Excision of a stem segement with 4-5 nodes without apex and roots and culture on liquid micropropagation medium as check (above) and on selective medium with fungus culture filtrate (below) 6. Growth of shoots derived from cultured nodes. 7. culture on solid fresh medium of apical buds taken from shoots insensitive to culture filtrte. 8. Growth of plantlet sresulting from apical buds. 9. Plant derived from shoots insensitive to culture filtrate and transferred into the plot.

Application of Restriction Fragment Length Polymorphisms and Genetic Mapping to Potato Breeding

M. W. Bonierbale, R. L. Plaisted, and S. D. Tanksley[1]

Introduction

Application of Molecular Genetic Mapping to Potato Improvement

The potato, as a crop of worldwide importance, with a vast, well-characterized but under-utilized germplasm pool, is an excellent candidate to benefit from the application of restriction fragment length polymorphism (RFLP)-based techniques of molecular genetics. Due to sensitivity to inbreeding depression, and genetic and cytoplasmic systems of incompatibility, the pure genetic line typically used for inheritance studies in other species are difficult to construct for potato. The cultivated potato (*Solanum tuberosum* L.) exhibits tetrasomic inheritance and higher order intragenic interactions than those of diploid species, which together with the quantitative inheritance of many valuable characters, complicates genetic analyses. Due to these biological features of the species, and to a lack of suitable markers, a classical genetic map has been developed for potato.

Due to a relatively high frequency of meiotic mutants leading to the development of unreduced gametes, the ploidy level of potato (4x) is readily manipulated, and although male sterility may interfere, it is possible to obtain intra- and interspecific diploid populations for breeding and genetic studies. In combination with this flexibility, linkage maps based on molecular markers provide new opportunities for applications in crop improvement. The codominant nature of molecular markers permits genetic analyses to be carried out in heterozygous stocks (Botstein et al., 1980; Soller and Beckmann, 1983; Helentjaris et al., 1985). Furthermore, as these markers detect variation at the DNA level, they are not genotype-environment subject to epistatic effects or to genotype-environment interaction. As favorable qualities for the application of molecular techniques, the potato has a relatively small genome (of low complexity), with considerable intraspecific DNA polymorphism (Gebhardt et al., 1989a). Reduction of ploidy level by induced parthenogenesis can proceed to the monoploid level, which yields homozygous stocks that can be used for restriction mapping. The potato can readily be transformed at the whole plant level, and is clonally propagated. Techniques for regeneration of potato plants from protoplasts are also well established.

[1]M. W. Bonierbale, R. L. Plaisted and S. D. Tanksley, Department of Plant Breeding, Cornell University, 252 Emerson Hall, Ithaca, NY 14853, U. S. A.

In 1987, Douches and Quiros monitored the segregation of isozyme markers in an interspecific diploid potato progeny, opening the door to more comprehensive genetic mapping based on molecular markers. In our laboratory, we have now developed a complete linkage map of potato-based RFLPs (Bonierbale et al., 1988), and several applications are in progress. Applications of RFLP maps for crop plant improvement and plant biology will stem from the association of markers with genes controlling traits of agronomic interest. RFLP techniques offer increased efficiency in selection of desirable genotypes in breeding programs and a means for isolating genes whose products are not known. The steps involved in moving toward these goals are: 1) assessing RFLP variability between breeding lines or among germplasm resources, 2) gene localization and fingerprinting for the identification of individuals carrying specific desirable traits under selection, and 3) development of physical maps of regions of the genome to which important genes have been localized, as a prerequisite to map-based cloning.

Concepts and Technical Aspects

Southern-blot analyses of restriction fragments generated by endonucleases allow rapid investigation of genomic differences among individuals. Since differences already present in individuals are used as the observed characters, it is not necessary to introduce markers, such as morphological mutants, into the populations under study. These techniques enable the development of genetic maps for species that have been difficult to study in the past. Shortages of available markers, difficulties encountered in making controlled crosses with ample population size or complicated inheritance, have impeded the development of genetic maps for organisms of scientific interest, including man, animals, and crop species.

As characters for studying genomic variability, RFLPs offer greater sensitivity in detecting intrinsic differences than do other molecular techniques such as isozyme analysis or immunology, which detect variation within gene-coding sequences. This is because the restriction site cleavages, which result in RFLPs, occur throughout the genome, in both the coding and the more abundant, less-conserved, non-coding regions. RFLPs can be found in virtually unlimited numbers between related individuals from the intergeneric to the intrapopulation level.

Under appropriate conditions, DNA cleavage by restriction enzymes occurs at specific palindromic sequences, and the sites of these sequences vary in their distribution throughout the genomes of related individuals due to evolutionary divergence or other genetic events. Two types of events which may create new or eliminate existing restriction sites between previously identical sequences are nucleotide substitutions and insertions/deletions. In populations resulting from sexual crosses between polymorphic individuals, Mendelian inheritance of RFLPs can be followed to study their genomic organization, and eventually, the genetic control of important traits via gene mapping.

To resolve RFLPs, DNA is extracted from individuals and digested with a restriction enzyme to generate fragments which are subsequently separated by gel electrophoresis and transferred to

a membrane. Cloned DNA sequences are radio-labelled and used as probes to identify fragments which are homologous to themselves by hybridization and autoradiography. An excellent description of the technical aspects of RFLP analysis can be found in Beckmann and Soller (1986).

RFLP maps have now been developed for several crop species, including maize, lettuce, rice, tomato, pepper, and potato. Each of these maps was developed by genetic analysis of RFLPs detected by DNA probes from the respective species, or, in the case of tomato,pepper, and potato by a common set of probes from one species. In developing these maps, probes from a DNA library are used to follow the segregation of restriction fragments contributed by parents of a sexual cross to their offspring. Data are accumulated from successive probings of restricted DNA from a single mapping progeny of about 70 plants, and analyzed to determine which probes reveal fragments that are frequently found in the same individuals, i.e. that are genetically linked. DNA clones that hybridize to cosegregating restriction fragments are designated as the RFLP loci in a linkage group, and map distances are determined by the frequency of recombination between them.

Linkage groups can be assigned to the chromosomes by a variety of methods. One of these techniques involves the use of trisomic series within which dosage effects can be seen with RFLPs representing sequence on each chromosome. This process was illustrated by McCouch et al. (1988) for the chromosomes of rice. Trisomic analysis and cosegregation of RFLPs with markers from a classical genetic map were used to assign the tomato RFLP linkage groups to its chromosomes. In the case of the RFLP map of potato, which was based on DNA clones from tomato, the high degree of conservation of chromosome content and gene order allowed the assignment of linkage groups to the potato chromosomes by their homeology with tomato (see next section).

Uses of RFLP Maps of Crop Species

RFLP maps of crop species are being utilized in studies of genome evolution and organization, and as tools for variety identification and genetic analysis of important traits. In crop improvement, RFLPs linked to genes of interest can be used as aids in the selection of individuals carrying particular genes, or to monitor the introgression of desirable genes among breeding lines.

Assessment of genetic variability and taxonomic relationships

Nuclear RFLPs are a valuable tool for measuring variation within and among plant species. As compared with other molecular methods, such as chloroplast DNA analysis, isozymes, and biochemical assays, they are more sensitive within the range of genotypes that are of interest in crop improvement. If mapped molecular markers are used to compare genotypes, whole genomes can be sampled at virtually any density for variation that is inherited biparentally. Measurements of RFLP variation are quantitative, additive, and can readily be related to genetic distances (Nei and Li, 1979).

RFLP profiles are useful as discrete characters for the description of varieties or genotypes. In a recent survey of North American potato cultivars, sufficient allelic diversity was detected with RFLPs and isozymes to demonstrate the utility of these markers in fingerprinting varieties and determining parentage (Douches, in preparation). A similar survey is being conducted of European cultivars in conjunction with genetic studies of RFLP loci in tetraploids (W. Powell, personal communication). Gebhardt et al. (1989a) have been able to distinguish many diploid potato lines currently being used in Europe for the development of tetraploid varieties by their RFLP fingerprints. This approach can also be used to monitor changes in allele frequencies in population under natural or artificial selection, or to characterize material for the generation of new alleles in response to certain selection pressures such as forced inbreeding and tissue culture (Rivard et al., 1989).

In addition to their use in the identification of genotypes, RFLP profiles provide a basis on which to measure distance between varieties or individuals. Quantitative measurements of variability within and between species based on this type of data will be useful for decision-making in germplasm collections and breeding programs seeking to utilize the relatives of cultivated species which possess valuable traits. Estimates of genetic distance based on RFLPs will provide independent data sets to address the many uncertainties that persist in the taxonomy of the relatives of crop species, and further applications can be seen in efforts toward the utilization of germplasm resources. RFLP surveys of *Brassica*, *Lycopersicon*, *Lens*, and *Solanum* accessions have recently been utilized to assess taxonomic relationships. Knowledge of RFLP variability among species is a prerequisite to their use in developing population in which markers for genes of interest can be identified for use as tags in genetic studies and/or during introgression into desirable genetic backgrounds by backcrossing.

Gene mapping and enhanced breeding efficiency

In crop plant improvement, there are a number of important single gene traits that are frequently transferred from one genetic background to another. Tight linkage between RFLP markers and desirable genes will permit the inference of the presence of these genes by assaying plants for the markers, facilitating early and perhaps multiple-trait screening of populations for individuals carrying important traits. In tomato, lettuce, and rice, researchers have found RFLP markers linked to major genes for resistance to insects or pathogens. In some of these cases, RFLPs have been used to scan the genomes of near isogenic lines (differing only in the trait of interest) for the chromosome segment introgressed from a donor parent which is responsible for the resistant phenotype (Young et al., 1988). Candidate markers are those which reveal a polymorphism between the susceptible and resistant isolines, and these are tested for fidelity in a progeny segregating for the trait. A more direct method of finding markers for genes of interest is to map the trait itself with respect to a set of markers from throughout the genome by linkage analysis within a progeny that segregates for the trait.

From the relatives of potato, there are single genes for resistance to pathogens which are desirable in cultivars. As the potato is highly heterozygous and intolerant of inbreeding, the isoline

approach to variety development or gene tagging is not appropriate. Tetraploid and diploid progenies segregating for virus and nematode resistance are being used in efforts to correlate RFLPs with single genes for these traits in potato.

The majority of traits of agronomic interest are controlled by the joint action and interaction of several genes, and markedly influenced by the environment. In addition to tagging major genes, RFLP markers can be utilized to detect genetic loci underlying such quantitative traits (Lander and Botstein, 1989). In tomato, RFLPs have been used to detect and measure the effects of several quantitative traits (Tanksley and Hewitt, 1988; Paterson et al., 1988). A large number of progeny from a backcross of a wild species accession to the cultivated *L. esculentum* were evaluated in field trials for the characters of interest, and for their genotypes at RFLP loci at regular intervals throughout the genome. Several regions of the genome were identified as loci controlling the traits under study. This analysis permitted the direct investigation of the number and nature of quantitative trait loci (QTLs) involved. Stocks with each of these QTLs alone and in various combinations have been identified so that their individual and interactive effects can be measured.

RFLP markers are currently being used to identify chromosomal segments from *S. brevidens* that are responsible for resistance to bacterial soft rot (*Erwinia* sp.) and potato leaf roll virus in hybrids with *S. tuberosum* derived by protoplast fusion (C. Williams, in preparation). In a separate effort with potatoes, sexual progenies from crosses between *S. tuberosum* and *S. berthaultii* are being developed to study quantitatively inherited insect resistance and several correlated traits with RFLPs. With these techniques, loci governing both desirable and undesirable traits from wild species can be identified and monitored during introgression, and recombination between foreign genomes can be observed. A breeding program has been aimed at introgressing these traits into the cultivated background by recurrent selection (R. L. Plaisted, personal communication), but difficulties have been encountered due to unfavorable linkages or limited recombination between the donor and recipient genomes. Once identified with traits of agronomic importance, RFLPs can be used in the selection of desirable recombinants, which contain only those segments of a donor genome that contribute to the trait under study, in the background of adapted genotypes. Parallel research with *S. berthaultii* is directed toward characterizing the gene products responsible for its insect resistance (J. Steffens, personal communication). Identification of gene products and gene localization by tight linkage with RFLP markers will lead to better understanding of the genetics, evolution, and biochemistry of important traits that are difficult to study by other means, and open the door to a new approach to ultimately clone the controlling genes.

Physical mapping and map-based cloning

Until now, the cloning of genes from plants has been limited to genes whose protein product is well characterized or those in which a genetically defined transposon is known to be present (Orkin 1986; Schmidt et al., 1987). For most of the agronomically important genes, these situation

do not yet apply, and none have been cloned from any crop species. RFLP mapping provides a way to clone genes about which only the effect on the phenotype is known (Tanksley et al., 1989). This approach requires the development of linkage maps with high populations of genetic markers. A project is now underway to place a large number (approximately 1,000) of RFLP markers on a molecular genetic map of tomato so that physical mapping of regions of interest can proceed. In further developing the potato map, considerable economy can be gained from the use of mapped tomato clones, as the conservation of linkage order between the two genomes allows accurate prediction of their map position in potato. With this approach, a selected region of the potato genome to which a gene of interest has been localized can be saturated with markers in a very short time, and physical mapping can proceed.

Once a high-density linkage map of a chromosomal region of interest is available, the next step involved in map-based cloning is the development of a physical map of the region, using Pulsed Field Gel Electrophoresis (PFGE). This technique allows the size fractionation of large DNA fragments, and, following hybridizations of Southern blots with genetically close RFLP markers, facilitates determination of the physical distance (in millions of base pairs, Mbp) between markers (Ganal et al., 1989). Although each cM in the potato RFLP map represents at least 1 Mbp of DNA, the large fragments from PFGE should contain more than one RFLP locus, or ideally, markers flanking a gene of interest. Techniques for PFGE of potato DNA have been utilized to develop a physical map of the region of the genome containing the patatin-coding genes. After a physical map has been constructed and a gene of interest has been localized to a particular DNA fragment, the entire region must be cloned for further characterization. A promising cloning system for this purpose is that of yeast artificial chromosomes (YACs) described by Burke et al. (1987). Finally, the presence of the gene of interest must be verified in cloned DNA. Most likely this will be done by transformation of the entire cloned region into the species of interest with the subsequent identification of the desired phenotype.

Genetic studies and breeding efforts toward potato improvement are complicated by heterozygosity and polyploidy, and techniques that promise to lead more directly to the identification, selection and production of desired genotypes are particularly attractive for such a crop. RFLP mapping, combined with the development of 2x breeding populations, promises to significantly advance potato genetics and provide opportunities for manipulating potato germplasm with a degree of precision not previously possible.

Development of the Potato Map

The first genetic map of potato, based on single-copy DNA probes from tomato, was constructed in a segregating cross between *S. phureja* and a *S. tuberosum* x *S. chacoense* hybrid (Bonierbale et al., 1988) (Fig. 1). This diploid progeny was selected for its high degree of polymorphism and prior characterization for isozyme loci (Douches and Quiros, 1987). The employment of tomato clones was suggested by the facts that potato and tomato are closely related in the family *Solanaceae*, and have nearly identical karyotypes with the same basic chromosome number of 12 and with significant nucleotide sequence homology. This high degree of DNA homology and the

use of tomato clones allowed the development of comparative maps between potato and the genetically well-characterized tomato.

By segregation analysis in the interspecific *Solanum* progeny, 134 segregating DNA and isozyme markers were mapped to the 12 potato chromosomes. The same markers were mapped in an interspecific tomato cross (Bernatzky and Tanksley, 1986), in which they spanned 94% of the genome. In comparing the chromosome content and linkage order between tomato and potato, the only rearrangements detected were four paracentric inversions involving three of the homeologous chromosomes; the remaining nine chromosomes were found to be entirely homosequential between the two genera at this level of resolution (Bonierbale et al., 1988). This high degree of conservation allowed the assignment of the linkage groups to specific chromosomes based on homology with tomato.

Although the potato and tomato genomes are nearly identical in karyotype, RFLP mapping revealed a significant difference in their genetic lengths. Recombination among the common set of markers in potato was approximately one-half of that observed in tomato (606 cM vs. 1189 cM). Double crossovers were extremely rare in the potato cross and a large proportion of the individuals failed to experience a single crossover in a majority of their chromosomes. A second linkage map of potato (Gebhardt et al., 1989b), based on a cross between diploid *S. tuberosum* clones demonstrated a genetic length of 690 cM, indicating that the observed amount of recombination relative to tomato is not cross-specific, but a general feature of potato species.

Genetic maps based on chromosome-specific molecular markers, such as those now available for potato, provide the ability to localize any cloned DNA sequence, including repeated sequences if they are polymorphic, in the genome. Through linkage analysis with RFLP markers, for example, the cloned genes for patatin storage protein have been mapped in potato and tomato.

Assessing Genetic Variability Among Germplasm Resources

Estimates of genetic distance based on RFLPs will provide independent data sets to address the many uncertainties that persist in *Solanum* taxonomy, and further applications can be seen in efforts toward the utilization of germplasm resources. The genetic resources of potato are vast, and many sources of desirable traits have been identified in related species. Quantitative measurements of variability within and between *Solanum* species will be useful for decision-making in germplasm collections and breeding programs seeking to utilize relatives of the cultivated potato. An RFLP survey of 90 *Solanum* accessions representing 18 species in which useful traits have been identified has recently been completed (M. Bonierbale et al., in preparation). RFLP variation within species was found to correlate very well with classical concepts of genetic diversity based on morphology, breeding behavior and geographic distribution (Fig. 2). A dendrogram of the calculated genetic distances between the species surveyed is presented in Figure 3. Knowledge of RFLP variability among species is prerequisite to their use in

developing populations in which markers for genes of interest can be identified for use as tags in genetic studies and/or during introgression into desirable genetic backgrounds by back-crossing.

In conclusion, RFLP techniques represent a potentially powerful tool for potato genetics and breeding. Unlike many other genetic tools, these techniques are amenable to use on outcrossing, polyploid species like potato. RFLP mapping, combined with the development of 2x breeding populations, promises to significantly advance potato genetics and provide opportunities for manipulating potato germplasm with a degree of precision not previously possible. Since RFLP maps now provide a close tie between potato and tomato genetics, we can also expect much cross feeding and utilization of information and resources between these two disciplines.

References

Beckmann, J. S. and M. Soller. 1986. Restriction fragment length polymorphisms and genetic improvement of agricultural species. Euphytica 35:111-124.

Bernatzky, R., and S. D. Tanksley. 1986. Toward a saturated linkage map in tomato based on isozymes and random cDNA sequences. Genetics 112:887-898.

Bonierbale, M. W., R. L. Plaisted, and S. D. Tanksley. 1988. RFLP maps based on a common set of clones reveal modes of chromosomal evolution in potato and tomato. Genetics 120:1095-1103.

Botstein, D., R. White, M. Skolnick, and R. W. Davis. 1980. Construction of a genetic linkage map in man using restriction fragment length polymorphisms. Am. J. Hum. Genet. 32:314-331.

Burke, D. T., G. F. Carle, M. V. Olson. 1987. Cloning of large segments of exogenous DNA into yeast by means of artificial chromosome vectors. Science 236:806-812.

Douches, D. S. and C. F. Quiros. 1987. Use of 4x-2x crosses to determine gene-centromere map distances of isozyme loci in *Solanum* species. Genome 29:519-527.

Ganal, M. W., N. D. Young, and S. D. Tanksley. 1989. Pulsed field gel electrophoresis and physical mapping in the *Tm-2a* region of chromosome 9 in tomato. Mol. Gen. Genet. 215:395-400.

Gebhardt, C., C. Blomendahl, U. Schachtschnabel, T. Debener, F. Salamini, and E. Ritter. 1989a. Identification of 2n breeding lines and 4n varieties of potato (*Solanum tuberosum* spp. *tuberosum*) with RFLP fingerprints. Theor. Appl. Genet. 78:16-22.

Gebhardt, C, E. Ritter, T. Debener, U. Schachtschnabel, B. Walkemeier, H. Uhrig, and F. Salamini. 1989b. RFLP analysis and linkage mapping in *Solanum tuberosum*. Theor. Appl. Genet. 78:65-75.

Helentjaris, T., G. King, M. Slocum, C. Siedenstrang, and S. Wegman. 1985. Restriction fragment polymorphisms as probes for plant diversity and their development as tool for applied plant breeding. Plant Mol. Biol. 5:109-118.

Lander, E. S. and D. Botstein. 1989. Mapping Mendelian factors underlying quantitative traits using RFLP linkage maps. Genetics 121:185-199.

McCouch, S. R., G. Kochert, Z. H. Yu, Z. Y. Wang, G. S. Khush, W. R. Coffman, and S. D. Tanksley. 1988. Molecular mapping of rice chromosomes. Theor. Appl. Genet. 76:815-829.

Nei, M. and W. H. Li. 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc. Natl. Acad. Sci. U.S.A. 76:5269-5273.

Orkin, S. H. 1986. Reverse genetics and human disease. Cell 47: 845-850.

Paterson, A. H., E. S. Lander, J. D. Hewitt, S. Peterson, S. E. Lincoln, and S. D. Tanksley. 1988. Resolution of quantitative traits into Mendelian factors by using a complete linkage map of restriction fragment length polymorphisms. Nature 335:721-726.

Rivard et al., 1989.

Schmidt, R. J., F. A. Burr, and B. Burr. 1987. Transposon tagging and molecular analysis of the maize regulatory locus *opaque-2*. Science 238:960-963.

Soller, M., and J. S. Beckmann. 1983. Genetic polymorphism in varietal identification and genetic improvement. Theor. Appl. Genet. 67:25-33.

Tanksley, S. D. 1983. Molecular markers in plant breeding. Plant Mol. Biol. Rep. 1:3-8.

Tanksley, S. and D. J. Hewitt. 1988. Use of molecular markers in breeding for soluble solids content in tomato: a re-examination. Theor. Appl. Genet. 75:811-823.

Tanksley, S. D., N. D. Young, A. H. Paterson, and M. W. Bonierbale. 1989. RFLP mapping in plant breeding: new tools for an old science. Biotechnology 7:257-264.

Williams, C. in preparation.

Young N. D., D. Zamir, M. W. Ganal, and S. D. Tanksley. 1988. Use of isogenic lines and simultaneous probing to identify DNA markers tightly linked to the *Tm-2a* gene in tomato. Genetics 120:579-585.

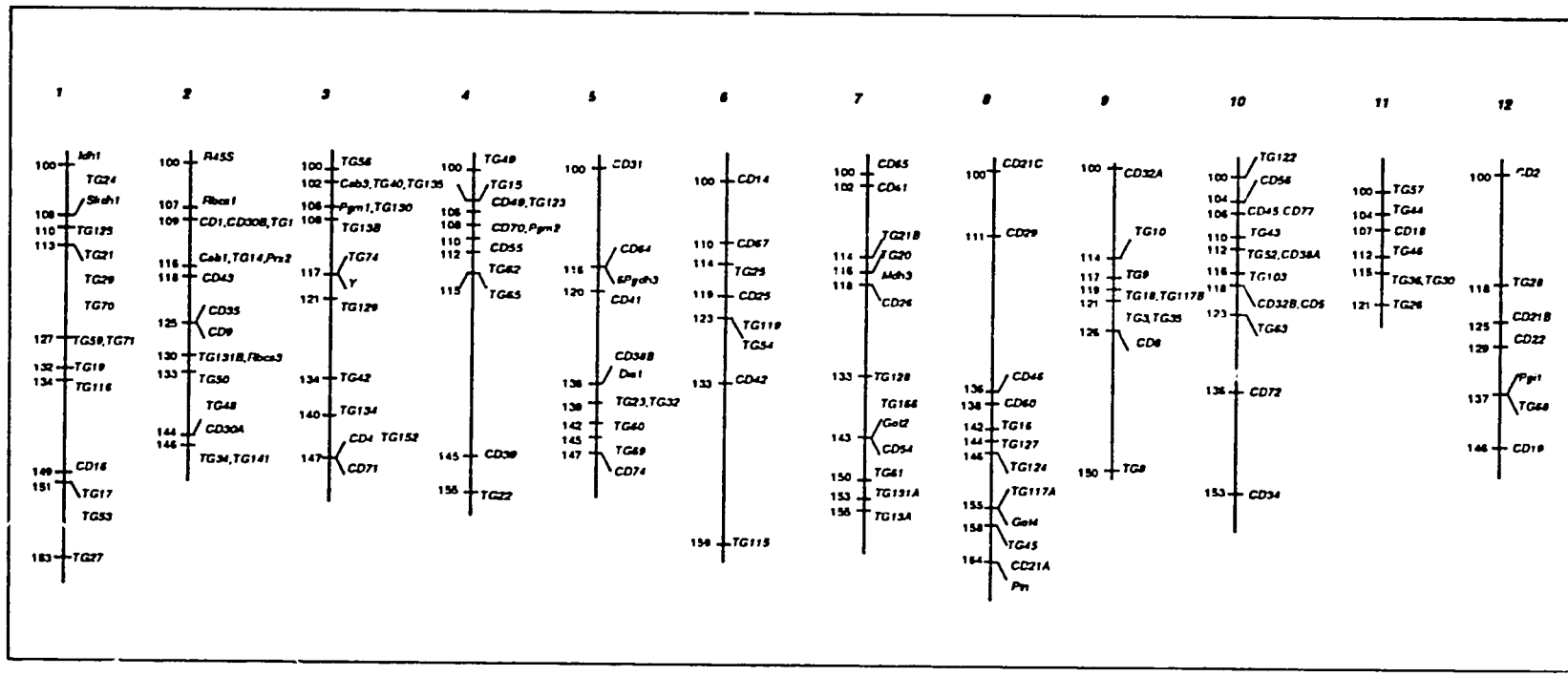


Figure 1. Molecular genetic map of potato constructed from the segregation of markers in a cross between *Solanum phureja* and a dihaploid *S. tuberosum x S. chacoense* hybrid. The majority of the markers (TG and CD-designations) are RFLPs detected by DNA probes from tomato. Due to the high degree of conservation of linkage order the 12 linkage groups in potato were assigned to chromosomes by homeology with the tomato genome (Bonierbale et al., 1988).

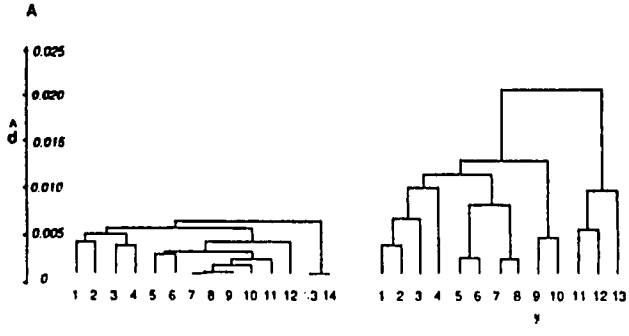


Figure 2. Estimates of genetic distance (vertical axis) among individuals within *Solanum* species based on RFLP variability at 27 loci distributed throughout the genome. A similarity index, the proportion of fragments shared, was calculated and transformed to an estimate of the frequency of base pair substitutions according to the formulas of Nei and Li (1979). A. Fourteen individuals representing *S. demissum*, a self-compatible allohexaploid species B. Thirteen individuals representing *S. chacoense*, a self-incompatible, diploid species (Redrawn from M. Bonierbale et al., in preparation).

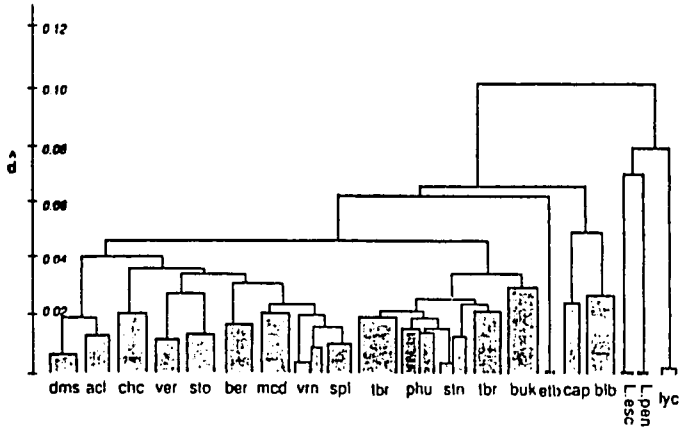


Figure 3. Estimates of genetic distance (vertical axis) among 18 *Solanum* and two *Lycopersicon* species. Calculations were performed as described for Figure 2. Height of histograms correspond to the maximum distance detected among an average of 10 individuals from each species.

Species-Specific DNA Probes in Introgression Breeding of Potato

C. Brown[1], B. Hall[2], M. Hwang[2], A. Ager[2], B. McConaughy[2], J. P. Helgeson[3]

Introduction:

Wild and primitive relatives of cultivated potato (ssp. *tuberosum*) comprise a reservoir of useful genetic variation that could be used for future variety improvement. There are several factors that discourage investment in programs that utilize wild or primitive germplasm. The first of these is the horticultural unsuitability of initial hybrids and early generation backcross segregants. Usually, there is discouragingly poor performance in numerous metric characters, absence of specific tuber shape and skin types from which to select, and sometimes, an unmanageably low harvestable yield.

A second caveat is the lack of ability to discern and control the processes by which an alien genome is disassembled and incorporated piecemeal into the cultivated genome. Presumably, in the numerous cases where genes from wild species have already been incorporated, this has occurred by retention of wild genes on wild chromosome segments. The chromosomes were then either incorporated whole or as fragments, resulting from breakage or recombination, and inserted into chromosomes of the cultivated genome. Furthermore, the execution of incorporation has been achieved by sorting through backcross segregants using pest, pathogen, and abiotic stress screens to aid selection.

The breeders' impression of progress toward a plant ideotype played an important role in selection. Clearly, use of alien germplasm is only undertaken under a long timeframe and when appropriate genetic variation is not present in the adapted gene pool. The problems that must be addressed in potato improvement, however, increasingly require genes outside of this intensively-bred adapted gene pool.

The question being addressed here concerns whether it would be possible to develop molecular markers that would provide a quantitative basis for selecting for backcross segregants that contain the least amount of alien genome while keeping the gene(s) of interest.

In the genomes of higher organisms, major classes of repetitive DNA are tandem repeats emanating from one or a few genetic loci and dispersed repetitive sequences, which occur singly, but scattered over many loci, in each case sandwiched in between different surrounding DNA

[1]C. Brown. USDA-Agricultural Research Service, Prosser, WA. U.S.A.
[2]B. Hall, M. Hwang, A. Ager, B. McConaughy. Department of Genetics, University of Washington, Seattle, WA.
[3]J. Helgeson, USDA/ARS, Department of Plant of Plant Pathology, University of Winconsin, Madison, WI, U.S.A.

contexts. The dispersed repeated elements themselves range in length from 200 to 5000 base pairs in length, and are found in nuclear DNA of higher plants and of many animal species. The utility of species-specific sequences stems from their horizontal dispersion from site to site within the DNA of a species. It has been possible to distinguish between DNA of different mammalian (Shih and Weinberg, 1982), primate (Daniels and Deininger, 1985), and cereal grain species (Flavell et al., 1977), by analysis of dispersed repeats unique to one species.

If repeated sequences unique to different taxa of *Solanum* spp. could be found, then the stage would be set to utilize them in breeding work to accelerate the introgression of traits from wild species. Typically, the incorporation process takes many years and consists of repeated crosses of successive segregants to advanced clones. Although it is a backcrossing operation in potato, there is no single recurrent parent, but rather a series of parents drawn from what should be called the recurrent gene pool. An actual example is shown in Figure 1.

This work was initiated at the Max-Planck Institute in Cologne, Germany, in the 1940s and pursued until the release of the first varieties in the mid-1970s. It is presumed that the vast majority of the allotetraploid *stoloniferum* genomes were discarded as selection toward the desirable plant type proceeded.

The availability of portions of this Ry_{sto} pedigree at the Max-Planck Institute provided the opportunity to select species-specific sequences. The objectives of this project, as it was originally conceived, were to find molecular markers closely linked to the Ry_{sto} gene. The approach was intended to be a means of finding linkage to genes of unknown biochemical function by following vestiges of the *stoloniferum* genome through the backcross generations. Supposedly, by the time the Ry_{sto} gene reached the BC7 generation cultivars of Ute or Roeslau it would be surrounded by a reduced envelope of hitchhiking *stoloniferum* chromosomes. Concomitantly, any uniquely *stoloniferum* DNA would most likely be contained within that envelope and linked to Ry_{sto} as a result of the probabilistic elimination of unselected *stoloniferum* genomes. The degree of linkage between marker and gene would depend on the actual mechanisms of incorporation and the distribution of the marker in the wild donor.

Approaches and Discussion of Results

Work on finding a *stoloniferum*-unique element was begun by Dr. Ben Hall in 1986, and involved the efforts of B. McConaughy and A. Ager. Repeated probes were selected by replicated colony hybridizations using whole unfractionated digested and 32P-labeled *stoloniferum* DNA as the probe. Several strategies were attempted to identify repeated elements present in *S. stoloniferum* but absent in cultivated potato. In the first strategy, nuclear DNA of cv. Russet Burbank served as "generic" cultivated potato DNA lacking infusion of wild species genes. Russet Burbank was used because it has an unusual origin. It was the great grand-offspring of a Chilean clone, Rough Purple Chili, introduced to the U.S. in 1851. Its ancestry predates 20th century efforts, which incorporated wild species related to *S. stoloniferum* as sources of disease resistance. Hence only elements which hybridized to genomic *S. stoloniferum* under hybridization conditions which

selected for high copy number, and not with Russet Burbank (i.e., generic cultivated potato) DNA were selected. Out of 3,000 colonies screened, only 3 were specific to *Solanum stoloniferum* and repeated.

In a second strategy, the *stoloniferum* DNA was enriched for elements unique to *stoloniferum* by a pre-incubation renaturation with nuclear DNA from Russet Burbank at a Cot of 200. After fractionation in a hydroxyapatite column to remove cross-reacting sequences, the eluted 32P-labeled single strand fragments were hybridized to a genomic library made from an advanced backcross member of the pedigree, 'Roeslau.' Hybridization with 32P-labeled *stoloniferum* DNA was again replicated under two conditions 1) with competitor DNA (from Russet Burbank) and 2) without. As before, colonies that had equivalent signal strength were candidates for uniqueness in *Solanum stoloniferum*. Southern blots displaying the hybridization pattern of selected clones hybridized to Hind III-digested nuclear DNA of several varieties, including Russet Burbank showed, in some cases, signals appearing at numerous fragment sizes on *stoloniferum* DNA, these were absent in Russet Burbank and other cultivars. In this experiment, 8 out of 500 colonies screened contained elements that were unique to *stoloniferum*. This was a higher yield than the above mentioned strategy, which did not include measures to enrich sequence specificity prior to colony hybridization. As expected, these sequences were rare, but were selected with moderate effort. This project did not succeed in finding a *stoloniferum* element that was transmitted through the Ry_{sto} pedigree above due to the inability to ascertain the exact accession that was the source of the Ry_{sto}. More importantly, however, it did demonstrate the existence and selectability of species-specific repeated elements in the case of one wild species, a positive result that encouraged the further pursuit of this research.

The need to use 'Russet Burbank' as a generic cultivar revealed a possible weakness in this approach. Species within the same taxonomic series are likely to share whole or partial genomes in common. The large number of wild species used in modern introgression breeding undertaken in the 20th century suggests that markers may not uniquely identify newly-introgressed germplasm if portions of related genomes are already in advanced breeding materials. This redundancy might be addressed, however, by creating the competitor/blocker DNA from the particular prospective cultivated recurrent breeding population into which the wild species traits have been or will be introgressed.

During 1989, a post-doctoral scientist, Dr. M. L. Hwang, working with Dr. Hall, selected repeated elements from the DNA of *Solanum brevidens* using techniques developed from the *stoloniferum* studies. *S. brevidens* is a non-tuber-bearing relative of potato with resistances to viruses and certain bacteria. It is not crossable with cultivated potato, hence protoplast fusion was used to make somatic hybrids with cultivated potato. Since *S. brevidens* is a diploid and the other fusion partner, R4, was a tetraploid, the initial hybrids were hexaploids. These were crossed once with the cultivar Katahdin to make pentaploid BC1 progeny and the BC1 was crossed again with 'Katahdin' to produce the BC2 progeny. This procedure is, in fact, how a breeder would proceed

to introduce a trait from *S. brevidens*. The pedigree was developed in J. P. Helgeson's laboratory at the University of Wisconsin (Helgeson et al., 1986). The crossing scheme is shown in Figure 2.

In the BC1, a whole haploid set of the *brevidens* genome (B) should be present. However, limited homology between B and T chromosomes will cause BC2 progenies to have different sets of B chromosomes because of the random loss of chromosomes due to limited homology and lack of pairing partners during gametogenesis of BC1 parents. We should see variation in the form of differing presence and absence among BC2 of restriction fragments. The Southern blots showing the hybridization pattern of one selected element were quite intriguing. This sequence hybridized with numerous fragment sizes on *brevidens* DNA, showing up as a smear. It was absent from the two *tuberosum* clones used in backcrossing and showed considerable variability in the BC2 segregants. The complexity of the pattern argued for dispersion among numerous chromosomes. This type of marker is a detector of wild species genomes in the segregants. It might be treated as a quantitative measure of wild species content in backcross segregants or specific fragments might be associated with co-segregating traits such as disease resistance. Jeffreys et al. (1986) demonstrated that it is possible to score Mendelian segregation of certain bands of repeated markers in the human genome. The results of *brevidens* project were a strong indicator of the robustness of this approach.

Conclusion

Studies concerning the use of species-specific DNA in breeding are in preliminary stages. It seems clear from the results presented above that species-specific markers can be found in *Solanum* spp. Although their analytical power is certainly less precise than single-copy markers of the potato gene maps now available (Bonierbale et al., 1988; Gebhardt et al., 1989), they may have a particular suitability in introgression studies in that relatively few probes can supply a sufficient level of information to aid the plant breeder in selection. Schemes that seek to bring highly reduced portions of alien genomes into the cultivated gene pool by somatic methods might find such markers of great utility to evaluate the success of the method. Any acceleration that might be achieved in the long introgression process is more likely to occur in the early generations of introgression when the quantity of the wild species genome is greatest and the potential impact of judiciously discarding wild species genome is highest.

References

Bonierbale, M. W., R. L. Plaisted, and S. D. Tanksley. 1988. RFLP maps based on a common set of clones reveal modes of chromosomal evolution in potato and tomato. Genetics 120:1095-1103.

Daniels, G. R. and P. L. Deininger. 1985. Repeated sequence families derived from mammalian tRNA genes. Nature 317:819-822.

Flavell, R. B., J. Rimpau, and D. B. Smith. 1977. Repeated sequence DNA relationships in four cereal genomes. Chromosoma (Berlin) 63:205-222.

Gebhardt, C., D. Ritter, T. Debener, U. Schachtschabel, B. Walkmeier, H. Uhrig, and F. Salamini. 1989. RFLP analysis and linkage mapping in *Solanum tuberosum*. Theor. Appl. Genet. 78:65-75.

Helgeson, J. P., G. J. Hunt, G. T. Haberlach, S. Austin. 1986. Somatic hybrids between *Solanum brevidens* and *S. tuberosum*: expression of a late blight resistance gene and potato leafroll resistance. Plant Cell Rpts. 53:212-214.

Jeffreys, A. J., V. Wilson, S. L. Thein, D. J. Weatherall, and B. A. J. Ponder. 1986. DNA "fingerprints" and segregation analysis of multiple markers in human pedigrees. Am. J. Hum. Genet. 39:11-24.

Shih, C. and R. A. Weinberg. 1982. Isolation of a transforming sequence from a human bladder carcinoma cell line. Cell 29:161-169.

S. stoloniferum (Yi) x 'Fruhmolle'

Breeding line (Yi) x 'Falke'

Breeding line (Yi) x 'Vera'

48.57/3 (Yi) x 'Aquila'

50.140/5 (Yi) x 44.1016/10

55.957/54 (Yi) x 8724

64.704 (Yi) x 44.1016/10

65.346/19 (Yi) x 'Gamma'

'Ute'(Yi) 'Roeslau'(Yi)

Figure 1. Pedigree for introgression of Ry_{sto}, controlling resistance to potato virus Y (PVY) from *S. stoloniferum* into cultivated potato by backcrossing into a recurrent gene pool consisting of several different varieties or breeding clones. Yi = imuune to PVY

brevidens = BB *x* *tuberosum* 1 = TTTT

F_1 = BBTTTT (F_1) *x* *tuberosum* 2 = TTTT

BC_1 = BTTTT (BC_1) *x* *tuberosum* 2 = TTTT

(partial B) + TTTT = BC_2

tuberosum 1 = R4, *tuberosum* 2 = 'Katahdin'

Figure 2. Development of BC2 progenies containing *S. brevidens* genome.

Use of RFLPs to Fingerprint North American Potato Cultivars

D. S. Douches, R. Freyre, and K. Hicks[1]

Profile

Forty cultivated potato varieties were probed with 9 probe/restriction enzyme combinations to access the utility of DNA restriction fragment length polymorphisms (RFLPs) to identify and characterize potato cultivars. RFLPs were assayed in total DNA digests by Southern blotting and hybridization with ^{32}P labelled probes from tomato genomic DNA, potato genomic and cDNA, along with a probe for pea rDNA. An initial screen of 3 genotypes X 7 restriction enzymes were used to identify the optimal probe/restriction enzyme combination for cultivar fingerprinting. Nine probe/restriction enzyme combinations resolved 50 polymorphic fragments and 24 fixed fragments among the cultivars. In addition to the presence and absence of bands, dosage differences were noted among some fragments. All varieties could be distinguished with a minimum of 2 probe/restriction enzyme combinations. The lack of genetic analysis, tetrasomic nature, and the extensive amount of intervarietal variation precluded any interpretation of the RFLP genotype from the phenotypic banding patterns of each probe. The value of these RFLPs versus isozyme markers for cultivar identification will be discussed.

Introduction

A problem facing potato breeders, seed growers, germplasm curators, and micropropagators is the difficulty in confidently distinguishing cultivars. Clonal identification has been traditionally based upon various combinations of morphological, phenological, and floral criteria along with accurate record-keeping to minimize misclassification. For example, potato cultivars can be grouped according to type of tuber skin, flesh and shape, or by foliage and floral characteristics. This method can be difficult and time-consuming and is prone to error because of subjective interpretation due to environmental modification. A close genetic relationship between cultivars can compound the problem. In addition, evaluating the array of distinguishing traits may not be possible at the time needed (i.e. field vs. storage).

Numerous attempts have been made to develop biochemical markers which could be utilized in varietal identification (Zwartz, 1966; Loescheke and Stegemann, 1966; Desborough and Peloquin, 1968; Staub et al., 1982). Olivier and Martinez-Zapata (1986) utilized isozyme markers

[1]D. S. Douches, R. Freyre, and K. Hicks, Department of Crop and Soil Sciences, Michigan State University

to fingerprint European potato cultivars and Douches et al. (1989a) did the same with North American cultivars. Recent advances in molecular biology techniques have led to the development of a new array of polymorphic genetic markers which has applications to germplasm characterization. These markers, referred to as restriction fragment length polymorphisms or RFLPs, have been used by Jeffries, et al. (1985) to discriminate between closely related humans. Considerable DNA polymorphism has also been detected for various crops such as maize (Evola, et al. 1986) *Brassica* (Figdore, et al. 1988), lettuce (Landry et al., 1987), soybean (Apuya, et al. 1988), rice (McCouch, et al. 1988), and potato (Bonierbale et al., 1988; Douches et al., 1989; Gebhardt et al., 1989a; 1989b).

With the advent of utility patents and proprietary rights sought and received for new releases, it is imperative that reliable, rapid, and efficient methods that can objectively discriminate potato cultivars continue to be developed. In this paper, we will examine the value of RFLPs as an objective means to characterize and discriminate potato cultivars. In addition, we will compare the utility of the RFLP markers to isozyme loci for varietal characterization of 40 tetraploid cultivars.

Materials and Methods

Forty cultivars which were analyzed for DNA RFLPs are listed in Table 1. The cultivars chosen for this study ranged from Garnet Chili, released in 1853, to A74114-4, which is currently being released as Frontier Russet (J. Pavek, personal communication). The varieties were also classified into 5 tuber types.

DNA Preparation, Digestion, Electrophoresis, and Blotting

Virus-free tubers of the 40 potato varieties were obtained from Dr. N. S. (Bud) Wright, Agriculture Canada. Total DNA was extracted from 10 g of fresh-leaf tissue from greenhouse plants as described by Guri and Sink (1988) and 6 g of DNA from each cultivar was digested with BamHI, EcoRI, HindIII, and XbaI, according to manufacturer's instructions (BRL). Digested samples were fractionated on 0.9% TBE agarose gels and Southern transferred to Zetabind (Cuno) nylon membrane according to Reed and Mann (1985).

Probe Preparation

The probes were randomly chosen for this study from four groups of probes maintained in the laboratory (Table 2). These groups are:

1. Polymorphic tomato genomic clone
2. Potato genomic clones
3. Potato cDNA, and
4. A pea rDNA clone (heterologous gene family).

Plasmids containing probe inserts (Table 2) were purified according to Birnboim and Doly (1979). Inserts were isolated by electroelution from agarose gels and then random-primed with 32p-dCTP (Feinberg and Vogelstein, 1983). The denatured probe was then added to a plastic bag containing two 20 X 16cm filters that were prehybridized 2-3 hours in 25 ml of 0.25M $NaHPO_4$, pH 7.2, 0.25M NaCl, 7% SDS, 10% PEG, 0.5% non-fat dry milk, 1mM EDTA, then incubated at 65°C. Filters were washed in 2XSSC, 1% SDS at room temperature for 20 minutes, then for 30 minutes in 2XSSC, 1% SDS at 65°C. The filters were then washed twice in 0.1XSSC, 1% SDS at 65°C, blotted dry, wrapped in plastic, and exposed to XAR-5 x-ray film (Kodak) with 1 intensifying screen (DuPont) for 1-5 days at 80°C. The filters were reused following a 30-minute wash in 0.4N NaOH, 0.1XSSC, 0.5% SDS at 42°C, then in 0.2M Tris pH 7.5, 0.1XSSC, 0.5% SDS.

Analysis of Polymorphism

To determine the optimal probe/restriction enzyme combination, each probe was hybridized to a membrane in which three diploid genotypes (84S10, 84SD22, and 84S11) were individually digested with seven restriction enzymes (EcoRI, HindIII, XbaI, DraI, EcoRV, BamHI, and SstI). The probe/restriction enzyme combination that exhibited the greatest fragment length variation between the genotypes was then used to examine polymorphism in the cultivars. Fragments that were polymorphic in a probe/restriction enzyme combination were assigned a number starting with 1 for the smallest fragment and increasing successively according to size. The presence or absence of each fragment was coded as a 1 or 0 respectively. The data was then transcribed to a database for an IBM compatible PC.

Isozyme analysis

The isozyme data was obtained from a larger survey of cultivars by Douches et al. (1989b). Horizontal starch-gel electrophoresis technique has been described previously (Douches and Quiros, 1988).

Results

Polymorphism was identified for all probes when digested by 7 separate restriction enzymes, however, each probe varied markedly in this characteristic (Table 3). TG23 was polymorphic in 6 of the 7 restriction digestions, whereas G12 only resolved variation in the EcoRI digestion. EcoRI was the most valuable enzyme to reveal variation (eight of 9 probes) but only 5 of the 9 probes were hybridized to EcoRI digests of the cultivars. The other probes were hybridized to HindIII (2), BamHI (1), and XbaI (1) digests which resolved more extensive variation for those probes.

Table 4 summarizes the level of polymorphism identified in the cultivated potatoes with these 9 probe/restriction enzyme combinations. A total of 50 polymorphic fragments (absence vs. presence) and 24 fixed fragments (identical in all genotypes) were observed. However, dosage differences were noted among some fragments, this observation was not taken into account for the analysis. All nine probe/restriction enzyme combinations showed polymorphism. The TG

probes resolved an extensive amount of polymorphic fragments (32 of the 50 polymorphic fragments). The probed TTC_1P_16 was polymorphic for only 2 of the 14 fragments identified with the probe. All asexually-derived cultivars were distinguishable by as few as 2 probe/restriction enzyme combinations in this study.

Discussion

The results from this study indicate that DNA RFLPs offer a means to characterize and discriminate cultivated potatoes. Identification of useful probes for cultivar fingerprinting was very feasible. Even though the probes were randomly chosen for this study, we detected polymorphism in at least one probe/restriction enzyme combination. This pattern observed for the probes could probably be attributed to the highly heterozygous nature of the potato cultivars. However, These probes differed substantially in their ability to detect RFLPs within the 50 potato varieties. The TG probes were valuable in that they hybridized to the greatest number of restriction fragments (35) and were also highly variable (32 of 35 fragments were polymorphic). These probes had been previously selected and utilized to develop a genetic map in potato (Bonierbale, et al.,1988) and may have biased the detectable variation in a positive manner. G12 and G17 each hybridized to only two fragments and were used directly in this evaluation following selection for low copy number.

At this time, horizontal starch electrophoresis offers an additional means to characterize and discriminate potato cultivars. Douches et al. (1989a) was able to uniquely fingerprint 97 sexually-derived cultivars with an array of 13 polymorphic isozyme loci. In Figure 1, a dendrogram was constructed from the isozyme data for the subsample of cultivars which were utilized for the RFLP analysis. This figure illustrates a number of important points about the electrophoretic technique. The isozyme data does not discriminate between sports or line selections of potato cultivars. In this case, Russet Burbank and Burbank have identical isozyme patterns. The isozyme data only examines 13 discreet DNA segments within the potato genome. In addition, electrophoretic analysis may resolve only 30% of the variation that occurs at the DNA level (Gottlieb, 1977). Meanwhile, all sexually-derived cultivars exhibited unique isozyme fingerprints. The ability of this set of genetic markers to discriminate these varieties can be attributed to the tetrasomic nature in combination with heterozygosity that is fixed through vegetative doubling. We also observed that the degree of genetic relationship can influence the number of isozyme differences between cultivars. Alaska Frostless, a pentaploid with one genome from *S. acaule*, was characterized by unique isozyme alleles and grouped out from the other cultivars, whereas siblings such as Early Rose and Beauty of Hebron only differ for one allozyme.

This study clearly indicates that RFLPs provide an additional set of genetic markers to be used in germplasm characterization. These genetic markers offer similar advantages described for isozymes such as:

1. Co-dominance
2. Neutral fitness
3. Non-epistatic interactions.
4. Lack of environmental influence, except RFLPs offer a supply of genetic markers which is practically limitless.

According to Bailey (1983), this class of genetic markers fits the basic criteria for utilization in varietal characterization. RFLPs have been shown in this and other studies (Bonierbale et al., 1988; Gebhardt et al., 1989a; 1989b) to exhibit

1. Environmental stability
2. Distinguishable intervarietal variation
3. Minimal intervarietal variation, and
4. Reproducible laboratory results.

Additional parameters should also be considered to determine the value of genetic markers (isozyme or RFLP) for fingerprinting cultivars. Factors such as safety (exposure to radiation and toxic chemicals), technical skill, cost (labor and supplies), and analysis time all favor the exploitation of isozyme markers for initial screening of genotypes. The strength of these markers is also based on our understanding of the genetics of each locus and its allelic diversity. As a result, isozyme analysis of the North American cultivars provided a set of data in which Douches et al. (1989a) examined the introgression of species-specific alleles and obtained estimates of clonal heterozygosity levels. The power of RFLP analysis lies in the ability to resolve a significantly larger number of genetic loci in specific genotypes. This greater resolving power will allow closely related genotypes to be examined more intensely. At this time we are not able to determine the genotype of the RFLP markers from their phenotype (banding pattern). As we develop a genetic understanding of the RFLP patterns at the tetraploid level with these and other probes, we envision RFLPs providing a means to examine the germplasm more critically.

References

Apuya, N. R., B. L. Frazier, P. Keim, E. J. Roth, and K. G. Lark. 1988. Restriction fragment length polymorphisms as genetic markers in soybean. Glycine max (L.) merrill. Theoretical and Applied Genetics 75:889-901.

Bailey, D. C. 1983. Isozymic variation and plant breeder's rights. *In* Tanksley, S.D., Orton, T. J. (eds.) Isozymes in plant genetics and breeding, part A. Elsevier, Amsterdam, pp. 425-440.

Birnboim and Doly. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acid Res. 7:1513-1523.

Bonierbale, N. W., R. L. Plastid, and S. D. Tanksley. 1988. RFLP maps based on a common set of clones reveal modes of chromosomal evolution in potato and tomato. Genetics 120:1095-1103.

Desborough, S. and S. J. Peloquin. 1988. Potato variety identification by use of electrophoretic patterns of tuber proteins and enzymes. American Potato Journal 45:220-229.

Douches, D. S. and C. F. Quiros. 1988. Additional isozyme loci in tuber bearing *Solanum*. *In* heritance and linkage relationships. Journal of Heredity 79:377-384.

Douches, D. S., K. Ludlam, and E. Varga. 1989. Electrophoretic survey of North American potato varieties. American Potato Journal 66:518.

Douches, D. S., N. Ludlam, and N. Linnel. 1989. The use of RFLPs and isozymes to fingerprint North American Potato Cultivar. *In*, The Proceedings of the First International Symposium on the Molecular Biology of the Potato (Abst.) (in preparation).

Evola, S. V., F. A. Burr, and B. Burr. 1986. The suitability of restriction fragment length polymorphisms as genetic markers in maize. Theoretical and Applied Genetics 71:765-771.

Feinberg, A. P. and B. Vogelstein. 1983. A technique for radio labeling DNA restriction endonuclease fragments to high specific activity Anal. Biochem. 132:6-13.

Figdore, S. S., W. C. Kennard, K. M. Song, M. K. Slocum, and T. C. Osborn. 1988. Assessment of the degree of restriction fragment length polymorphism in *Brassica*. Theoretical Applied Genetics 75:833-840.

Gebhardt, C., C. Blomendahl, U. Schachtschabel, T. Debener, F. Salamini, and E. Ritter. 1989. Identification of 2n breeding lines and 4n varieties of potato (*Solanum tuberosum*, ssp. *tuberosum*) with RFLP-fingerprints. Theor. Appl. Genet. 78:16-22.

Gebhardt, C., E. Ritter, T. Debener, U. Schachtshabel, B. Walkemeier, H. Ubrig, and F. Salamini. 1989. RFLP analysis and linkage mapping in *Solanum tuberosum*. Theoretical Applied Genetics 78:65-75.

Gottliela, L. D. 1977. Electrophoretic evidence and plant systematics. Annals of Missouri Botanical Garden 64:161-180.

Guri, A. and K. C. Sink. 1988. *Agrobacterium* transformation of eggplant. J Plt Physiology. 133:52-55.

Jeffreys, A. J., V. Wilson, S. L. Thein. 1985. Hypervariable "mini-satellite" regions in human DNA. Nature 314:67-73.

Landry, B. S., R. Kesseli, Hei Leung, and R. W. Michelmore. 1987. A genetic map of lettuce (*Lactuca sativa* L.) with restriction fragment length polymorphism, isozyme, disease resistance and morphological markers. Genetics 116:331-337.

Loescheke, V. and H. Stegemann. 1966. Proteine der Kartoffelknollen in abhängigkeit von Sorte und Virosen (Polyacrylamidelektrophorese). Phytochemistry 5:985-991.

McCouch, S. R., G. Kochert, Z. H. Yu, Z. Y. Wang, G. S. Khush, W. R. Coffman, and S. O. Tanksley. 1988. Molecular mapping of rice chromosomes. Theoretical and Applied Genetics 76:815-829.

Olivier, J. L. and J. M. Martinez-Zapata. 1985. A genetic classification of potato cultivars based upon allozyne patterns. Theoretical and Applied Genetics. 69:305-311.

Reed, K. C. and D. A. Mann. 1985. Rapid transfer of DNA from agarose gels to nylon membranes 13(20):7202-7221.

Staub, J. E., L. J. Kuhns, P. Grun, and B. May. 1982. Stability of potato tuber isozymes under different storage regimes. Journal of the American Society for Horticultural Science. 107(3):405-408.

Zwertz, J. A. 1966. Potato varieties and their protein electropherogram characteristics. European Potato Journal 9:111-128.

Table 1. Tetraploid cultivars examined for RFLPs.

Cultivar	Year of Release	Type[a]
White Rose	1893	long
Rural NY	1888	round
Green Mountain	1885	round
Russet Burbank	1914	russet
Burbank	1876	long
Early Rose	1861	long
Irish Cobbler	1876	round
Beauty of Hebron	1878	red
Early Ohio	1871	round
Garnet Chili	1853	red
Bliss Triumph	1878	red
Katahdin	1932	round
Sebago	1938	round
Onaway	1961	round
Kennebec	1948	round
Red LaSoda	1948	red
Red Pontiac	1949	red
Norland	1957	red
Chieftain	1966	red
Spunta	?	round
Rosa	1980	round
Conestoga	1982	round
Desiree	?	red
Alaska Frostless[b]	1967	round
Yukon Gold	1980	round yellow
Saginaw Gold	1988	round yellow
Bintje	1910	round yellow
Belchip	1979	round
Chipbelle	1981	round
Atlantic	1978	round
Wauseon	1967	round
Superior	1961	round
Norgold Russet	1964	russet
Lemhi Russet	1981	russet
Nooksack	1973	russet
Butte	1978	russet
A74114-4	1988	russet
Krantz	1988	russet
NDD277-2	1988	round
Ontario	1946	round

[a] long = long shape with white flesh
russet = russet skin with white flesh
round = round shape with white flesh
red = red skin with white flesh
round yellow = round shape with yellow flesh

[b] pentaploid (2n=60)

Table 2. Polymorphic probes utilized for RFLP fingerprinting

Probe	Restriction Enzyme Digestion	Probe Source	Institution
TG26	EcoRI	Tomato genomic	Cornell
TG24	EcoRI	Tomato genomic	Cornell
TG23	HindIII	Tomato genomic	Cornell
TG16	HindIII	Tomato genomic	Cornell
pHA2	EcoRI/BamHI	Pea rDNA	NCSU
G12	EcoRI	Potato genomic	MSU
G17	EcoRI	Potato genomic	MSU
T_8P_423	EcoRI/BamHI	Potato genomic	MSU
TTC_1P_16	XbaI	Potato cDNA	MSU

Table 3. Survey of DNA polymorphism detected by various restriction enzymes.[a]

Probe	EcoRI	HindIII	BamHI	XbaI	EcoRV	DraI	SstI
TG26	+[b]	+	+	+			+
TG24	+	+		+	+	+	
TG23	+	+	+	+	+	+	
TG16		+		+	-	-	-
pHA2	+		+			+	
G12	+				-	-	-
G17	+		+	+	-	-	-
T_8P_423	+		+		-	-	
TTC_1P_16	+		+	+	-	-	

[a]Based upon three diploid genotypes.
[b]'+' indicates polymorphism was detected, '-' indicates no examination available.

Table 4. Levels of polymorphism for various probe/restriction enzyme combinations.

Probe	Restriction Enzyme	Polymorphic Fragments	Fixed Fragments
TG26	EcoRI	10	2
G12	EcoRI	2	1
pHA2	EcoRI	8	4
G17	EcoRI	2	0
TG23	HindIII	8	0
TG16	HindIII	5	0
T_8P_423	BamHI	4	4
TG24	EcoRI	9	1
TTC_1P_16	XbaI	2	12

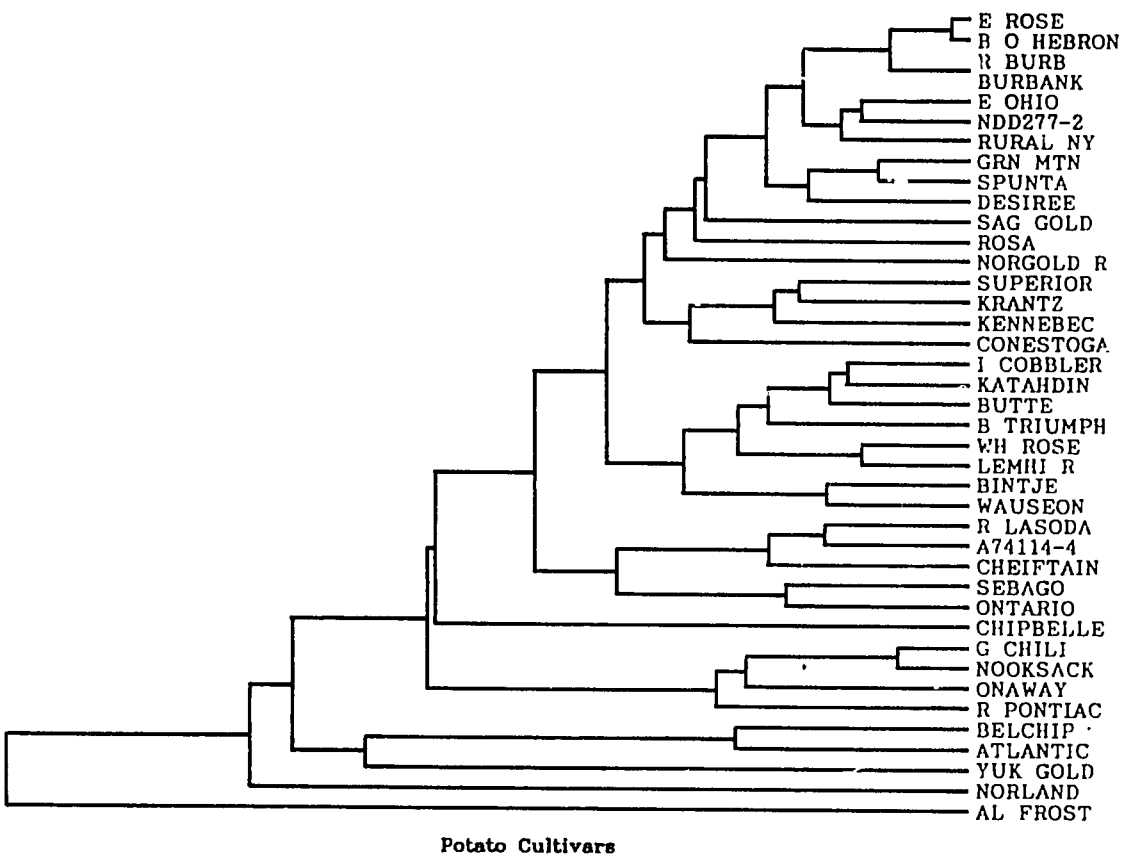


Figure 1. Segmation of potato cultivars based upon 13 isozyme loci.

The Andean Biotechnology Program (PAB) of the Andean Development Corporation (CAF)

M. Tejada[1]

The Andean Development Corporation CAF (Corporacion Andina de Fomento) is the development bank of the Andean Pact countries. Its shareholders are the five member countries of the subregion (Bolivia, Colombia, Ecuador, Peru, and Venezuela). Despite the fact that it started 20 years ago with little capital, its current authorized capital amounts to US$1 billion and, by a recent decision made by the Presidents of the member countries, necessary steps are being taken to increase it to US$2 billion.

CAF has two important roles to play within the Andean context:

1. To finance the industrial and, in general, productive development of the Andean countries. For this purpose, CAF provides loans to the governmental and private sectors of its member countries.

   Last year, CAF-financed projects and foreign trade activities amounted to US$400 million in all the five member countries.

2. To promote integration among its five member countries and to contribute to the improvement of the standard of living of the Andean population.

   Accordingly, CAF pledges non-reimbursable funds for agricultural and industrial productivity programs, funds for multinational enterprises, as well as support for the improvement of small-scale industry and the development of Biotechnology.

Currently, CAF has allocated $12 million to implement these non-reimbursable assistance programs. Such funds consist of a percentage of the annual profits obtained by CAF's banking activities. In other words, the funds are supplied by the Andean countries themselves.

In 1987, through the initiative of CAF's Executive President, Dr. Galo Montaño, the Andean Fund for Biotechnology Development was established, with an initial allocation of US$1 million of non-reimbursable funds.

The Andean Biotechnology Program (Programa Andino de Biotecnología, PAB), which is financed by the Andean Biotechnology Fund, is the basis for the specific action plan conceived by CAF to foster the development of Biotechnology and Genetic Engineering in the subregion.

[1]M. Tejada, Director, Andean Biotechnology Program, Coorporación Andina de Fomento (CAF), Venezuela

PAB was reviewed and approved by the representatives of the public and private sectors of the five member countries.

PAB's activities began in 1988 and have generated great interest in the countries. As a consequence, CAF is receiving a growing number of financial applications. The initial allocation of US$1 million was spent in projects developed during 1988, the first year of activities. An annual fund of US$1 million was allocated for 1989 and 1990 respectively, and it is hoped that this will be continued during successive years.

Why should a multinational bank decide to undertake a Biotechnology program?

This is a question which is frequently asked.

First of all, CAF, as an Andean development bank, seeks to contribute to the introduction of biotechnology in its member countries, in order to enhance the activities related to agriculture and food, as well as those related to the industrial and health sectors.

Secondly, as a credit agency, through PAB activities, CAF sponsors the creation of new agricultural, pharmaceutical, and industrial enterprises which would benefit from its conventional loans.

PAB has designed a global strategy in order to create an adequate technological capacity in the subregion and to link this capacity with the productive sectors.

CAF's program has three main fields of action:

1. Research projects
2. Industrial development
3. Training and technology transfer

The first area deals with financing applied research projects submitted by public or private enterprises for non-reimbursable funding. In keeping with PAB's objective for development of food and agriculture, production units involved in in vitro propagation of plants are being promoted. Therefore, priority is assigned to projects whose results may be quickly available to the agricultural sector. For this reason, the creation and improvement of tissue culture laboratories were immediately supported by CAF.

Twenty-one out of the thirty-one projects supported by CAF through grants deal with biotechnology as applied to plant micropropagation.

It must be emphasized that seven of the agricultural biotechnology projects, already implemented or about to be implemented, deal with the production of potato planting material. Such projects are aimed at obtaining certified seed. One of them includes a germplasm bank for Andean tubers. Two additional projects in sweet potato and *Ullucus tuberosus* are being implemented.

The present aim is to continue a strategy to develop a planned organization of new potato micropropagation units and, with the setting up of public or private enterprises, to produce certified potato seed in the five subregional countries. As a banking organization, CAF does not have experts or technical facilities of its own. Therefore, we have asked for the cooperation of specialized institutions such as CIP and CIAT (International Center of Tropical Agriculture), in order to achieve our objectives.

In CIP's collection a great number of the applied research projects financed by CAF deal with products under CIP's control. Thanks to CIP's support (which has been all-important since it has included global advisory activities, specific technical assistance and personnel training), CAF has been able to outline a strategy that has already been implemented and that we think will succeed.

CAF, in addition to producing the above-mentioned products, has tissue culture projects in cassava, cocoa, banana, other fruits, and in ornamental and horticultural plants. In general, all of these projects fulfill one of CAF's main goals, namely the transfer of the selected material from the research centers to the farmers or to agroindustry.

CAF gives support in the form of technical assistance and donations of some laboratory equipment to private firms and associations in agroindustry which are expected to cover the costs of personnel and of operation; in the case of support given to national laboratories, all costs are financed by CAF.

Some examples of our assistance rendered to private enterprises can be found in Peru (pineapples), Ecuador (bananas and roses) and Bolivia (strawberries and potatoes).

Another main area promoted by CAF is the organization of high-tech projects, aimed at developing molecular biology and genetic engineering techniques for propagation of plants, in the subregional research centers. Such activities were not undertaken in the laboratories of the Andean region before CAF's program began.

In a short period of time, and thanks to the generous contribution of Dr. Jesse Jaynes, for the first time in the subregion, we have attained positive results in obtaining potentially frost-resistant transgenic potato plants.

This work was performed in the Biology Laboratory of the Universidad Central de Venezuela.

A cassava genetic engineering project has been initiated in Venezuela at IDEA (International Institute of Advanced Studies) with the cooperation of CIAT. This project aims at ultimately reducing the cyanide content of bitter cassava species, and will be simultaneously supported by cassava regeneration technologies to be developed at the Universidad Nacional Mayor de San Marcos, in Peru.

Other projects are being conceived for obtaining high yield and resistance to fungi. This activity will be coordinated by two Venezuelan research centers and a subregional scientist trained by Dr. Jaynes, who will be project Leader.

Another genetic engineering project dealing with sweet potato will be implemented, coordinated by CIP and Dr. Schilde's laboratory at Tubingen. This project will be financed by CAF and the EEC on a cost-sharing basis, and will develop protoplast techniques for the direct transfer of genes to obtain resistance to *Erwinia carotovora* and *Pseudomonas solanacearum*.

We are also evaluating a project for adoption of rDNA technologies for *Passiflorae* spp. in Colombia as well as one for sweet cucumber in Ecuador.

In accordance with CAF's policy, the first phase of genetic engineering activities is aimed at undertaking several research projects on genetically-altered plants. Initially, the subregional laboratories will deal only with regeneration and verification technologies. We hope to depend upon the genetic constructions readily available from international laboratories.

Simultaneously, CAF is organizing a research unit in Venezuela, with focus groups on molecular biology and plant genetic engineering; this unit will serve as an information center for all of the Andean subregion.

As regards CAF's activities in the industrial sector, actions are being initiated in the development and application of modern diagnostic methods, keeping in mind our purpose of transferring the laboratory results to the commercial sector. A recent achievement is a Chagas disease diagnostic kit, which has already been commercially promoted by CAF. An enzyme based on the manipulation of *E. coli* is about to be developed by another Venezuelan laboratory.

CAF has also given priority to fermentation and extraction technologies. Projects dealing with vegetable dyes, organic acids, pectins, champignons, as well as high investment projects for penicillin, lysin and vitamin-producing plants are being promoted by CAF, which is undertaking feasibility studies.

As a consequence of the above, plans are being made to set up an agricultural and industrial complex in Venezuela to produce enriched cassava beast for human and animal consumption. CAF has done feasibility studies that include maintenance of a 2,000-hectare cassava plantation, whose basic propagation material will be obtained by *in vitro* techniques. The total investment of this project amounts to US$10 million, out of which 3 million will be provided by CAF to the enterprise, as a loan.

A second project deals with the production of blood plasma and human blood products for an enterprise founded by the Venezuelan Scientific Research Institute (IVIC) and the Venezuelan Government. CAF granted a US$5 million loan for the plant, which is currently under construction and which will be the first of its kind in the subregion. A third project deals with the

production of nitro-bacterium-based fertilizers in Venezuela. This project is to be implemented by a private firm.

Finally, as a main objective, PAB has included in its strategy the training and technology transfer at intra and extra-subregional levels. One of the main purposes of this program is to create a coordinated network among Andean scientists and research centers.

Such integration has been achieved through concrete actions and not through formal agreements, which, according to our experience, rarely succeed. Installation of new laboratories, technical assistance and training have improved a great deal, due to cooperation and exchange of scientists of the subregion.

Concerning training, up to now, CAF has directly organized or contributed to more than a dozen courses dealing with plant tissue culture and genetic engineering. It has also facilitated individual training of more than forty technicians inside and outside the subregion, mainly in tissue culture techniques. Recently, CAF financed the postdoctoral specialization of two molecular biologists previously involved in human medicine and who have shown an aptitude for genetic engineering of plants. Currently, these two scientists are the only ones working in the national research centers of the subregion.

This high-level specialization program will be continued in the future, and we will try always to balance the number and level of the scientists selected, and to assure the continuance of the research, in keeping with the specific needs of each of the Andean countries.

Up to now, PAB has worked with CAF's own resources, which amount to US$2 million of already-committed expenses.

The response of the countries to CAF's Biotechnology Program has been very positive and demands for new projects are increasing daily. By the end of 1990, there will be 50 projects approved or under way. It will become imperative to strengthen the cooperation of international research centers, laboratories and other organizations to foster CAF's efforts and to meet the demands of the Andean countries.