IICA

INSTITUTO INTERAMERICANO DE COOPERAÇÃO PARA A AGRICULTURA — OEA —

Consultant Final Report
IICA/EMBRAPA-PROCENSUL II

IMMUNOCYTOCHEMICAL LOCALIZATION OF TRANSGENIC PROBLEMS IN PLANT TISSUES

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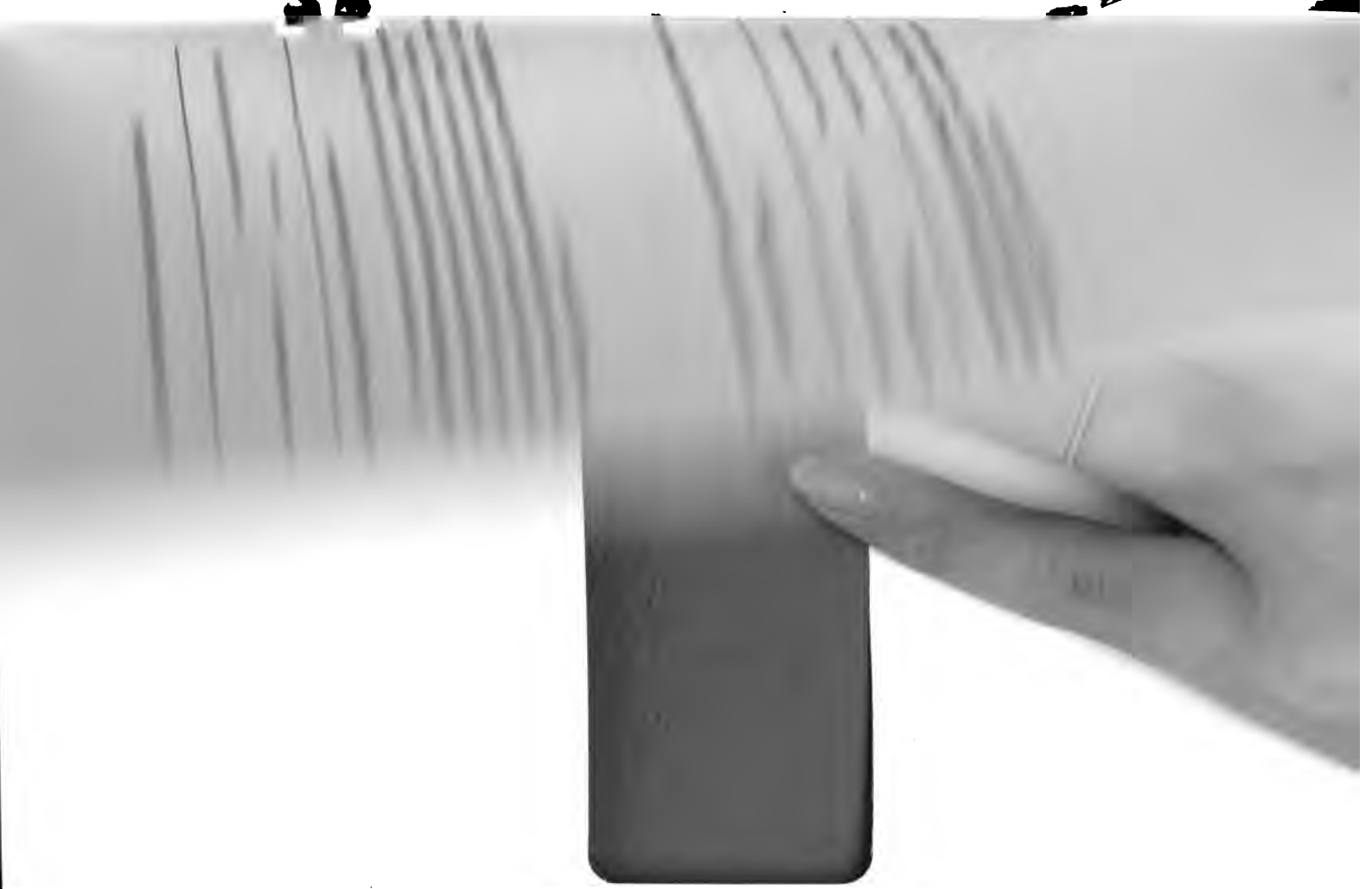

REPORTS UNDERTAKEN WITH THE CONSULTANT'S PARTICIPATION

Title of publication or report
bibliographic identification

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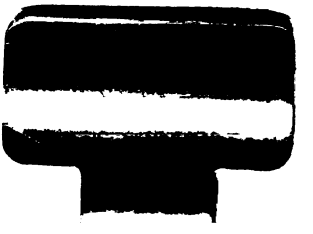

Consultant Final Report
IICA/EMBRAPA-PROCENSUL II

IMMUNOCYTOCHEMICAL LOCALIZATION OF TRANSGENIC PROBLEMS IN PLANT TISSUES

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IMMUNOCYTOCHEMICAL LOCALIZATION OF TRANSGENIC PROBLEMS IN PLANT TISSUES

IICA
MIS A4/BR-88-011
Harris, Nicholas
Immunocytochemical localization of transgenic problems in plant tissues. - Brasília, Brasil: Instituto Interamericano de Cooperação para a Agricultura, 1988.
15 p. (IICA - Publicações Miscelâneas, A4/BR-88-011)
ISSN 0534-0591

1. Pesquisa - Biotecnologia - Localização problemas transgenicos - Cultura de Tecidos - Brasil. I. Título. II. IICA (Brasil). III. Série.

AGRIS A50;
F30
CDU 581.176.001.5(81)

APRESENTAÇÃO

A reprodução e difusão dos Relatórios de Consultores, no âmbito restrito das Diretorias das Unidades do Sistema Nacional de Pesquisa Agropecuária, vinculado à EMBRAPA, tem como objetivo principal o de divulgar as atividades desenvolvidas pelos consultores e as opiniões e recomendações geradas sobre os problemas de interesse para a pesquisa agropecuária.

As atividades de consultoria são realizadas no âmbito do Projeto de Desenvolvimento da Pesquisa Agropecuária e Difusão de Tecnologia na Região Centro-Sul do Brasil-PROCENSUL II, financiado parcialmente pelo Banco Interamericano de Desenvolvimento-BID e a EMBRAPA conforme os contratos de Empréstimo 139/IC-BR e 760/SF-BR, assinados em 14 de março de 1985 entre o Governo brasileiro e o BID.

As opiniões dos consultores são inteiramente pessoais e não refletem, necessariamente, o ponto de vista do IICA ou da EMBRAPA.

A coordenação dos Contratos IICA/EMBRAPA agradeceria receber comentários sobre estes relatórios.

Horacio M. Stagno
Coordenador Contratos IICA/EMBRAPA

INTER-AMERICAN INSTITUTE FOR COOPERATION ON AGRICULTURE
IICA/EMBRAPA CONTRACT

CONSULTANT FINAL REPORT

1. Consultant's full name: Nicholas Harris
2. Specialist in: Botany
3. Title of IICA Project: 2.SB.3.

4. EMBRAPA Program for which consultancy is provided:

PROGRAMA : PROCENSUL II

SUB-PROGR: IV- RECURSOS GENETICOS E BIOTECNOLOGIA

IICA Project Activity Code: 2.SB.3.05	Administrative Code: R 4884 B1B 03105
Title of Activity of IICA Project corresponding to this consultancy	Cooperation with EMBRAPA on research and applications of genetic resources, biotechnology and biologic control of plagues, diseases and weeds.

CONSULTANT CONTRACT PERIOD	DUTY LOCATION (Center)
April 11 to 21., 1988	CENARGEN
CONTRACT EXTENTION PERIOD (If any)	DUTY LOCATION (Center)

5. Financial support:
British Council support for air fares UK-Brasília-UK.
Donation of reagents by the consultant to the value of US$ 1,500,00
Detailed in appendix I.

6. ACTIVITIES UNDERTAKEN BY THE CONSULTANT AND RESULTS

6.1 RESEARCH DONE UNDER DIRECT RESPONSIBILITY OF THE CONSULTANT

Research activities developed	Results Achieved
6.1.1. Methodology for preparation of Nicotiana seeds for examination by optical and electron microscopy.	Small seeds such as those of (transgenic) Nicotiana present various problems regarding preparation for histological and ultrastructural studies. These were discussed and methods described and carried out successfully by CENARGEN staff to overcome such difficulties.
6.1.2. Histological analysis of Nicotiana seed structure and development.	The patterns of tissue development in Nicotiana seeds were examined. Particular attention was drawn to (i) the differential rates of development in the endosperm and embryo tissues. (ii) specific identification of cotyledones, hypocotyl and radicle, apices and protovascular tissues.
6.1.3. Cytological analysis of protein body formation in endosperm and embryo tissues of developing Nicotiana seeds.	The deposition of seed storage proteins and the different processes in the endosperm and embryo tissue were described and illustrated using samples prepared for optical and electron microscopy.
6.1.4. Methodology for immunocytochemical analysis. A. For optical microscopy.	Experimental protocols were described and discussed and carried out, under the supervision of the consultant, to demonstrate in the optical microscope the immunocytochemical localisation of specific proteins. The method used involved localisation of the primary antibody, bound to antigens in resin embedded tissue, by use of a secondary goat-anti rabbit IgG conjugated to colloidal gold. The latter was subjected to silver intensification to permit visualisation of the markers.
B. For electron microscopy.	The method developed in CENARGEN utilizes the recognition of specific epitopes on/in tissue embedded in the hydrophobic resin LR White. Primary antibody distribution is then visualised by reacting with a secondary antibody conjugated to colloidal gold, with contrast of the specimen controlled by the extent of uranyl acetate staining. The methods although apparently straightforward, do require "fine-tuning" for each specimen in order to optimise the signal to background of immuno staining. The various approaches to such optimisation were described and variations tried in practical work.

Research activities developed	Results Achieved
6.1.5. Immunocytochemical analysis of the expression of transgenic 2S protein in seeds of transformed Nicotiana.	The experimental protocols described in 6.1.4. were used in an analysis of the distribution of the Brazil nut 2S high methionine protein in seeds of transformed Nicotiana. Samples were examined from a range of transgenic plants produced by both the Agrobacterium and protoplast methods. Preliminary results from seeds of the various transgenic plant gave positive immunolocalisation of the 2S protein at both optical and electron microscope levels. Unfortunately these results could not be confirmed during the period of the consultancy because of failure in the electron microscope prior to examination of the control (non-transformed) tissues. Optical microscopy studies did indicate a possible low level of non-specific binding but, because of the low level, this can only be confirmed by Em work. Discussions were held regarding the different routes to overcome any possible non-specific binding; in the consultant's laboratory the pretreatment of primary antisera with extract from non-transformed tissues has proved the simplest and most effective route, and protocols for this approach, as well as others including differential "blocking" and/or washing, have also been described. Further work on the transformed and non-transformed Nicotiana samples will be undertaken in both the CENARGEN and consultant's labs.
6.1.6. Presentation of results from immunocytochemical studies.	The presentation of results was discussed, particulary with regard to (1) the choice of appropriately sized gold and (2) the balance of tissue staining and the contrast levels suitable for both slides and prints. The use of multigrade printing paper was demonstrated, with material and filters donated to the project.

6.2 SUPPORT TO RESEARCH UNDERTAKEN BY OTHER EMBRAPA RESEARCHERS

Research activities developed	Results achieved

As well as interactive discussions with many of the senior EMBRAPA staff involved in the 2S transformation programme, the consultant also discussed the possible solutions to technical problems involving a variety of specimen preparations including eg insect eggs, fungal spores, embryo tissues etc. Specific detailed protocols have been suggested and literature references have either been provided already or will be forwarded from UK.

6.3 TRAINING ACTIVITIES DEVELOPED BY THE CONSULTANT

Date	Training subject matter	Type of event*	Number of beneficiaries	
			From EMBRAPA	From other institutions
10/04 to 19/04/88	Detailed analysis of specimen preparation techniques for histological and immunocytochemical study of plant tissues.	Practical demonstrations and discussions (Further detailed in 6.4.)	Full-time: 1 EMBRAPA	Part time 1 UNB
18/04/88	Integration of methods for the study of plant development and differentiation.	Seminar		At UNB: 20 + from other institutions.

* Short courses, seminars, conferences, etc.

6.4 IN-SERVICE TRAINING PROVIDED BY THE CONSULTANT

In-service training subject matter	Names of counterparts

A major part of the consultancy was devoted to in-service training of Eliana Santana (EMBRAPA/CENARGEN). The consultant demonstated and supervised practical techniques required for subsequent use by the biotechnology group for analysis of their transgenic plant material. At each stage crucial parameters were described and discussed. Eliana showed as excellent willingness to learn and adapt techniques and was keen to spend time outside of normal work hours in assisting the consultant in the research part of the consultancy.

6.5 ACTIVITIES IN SUPPORT OF RESEARCH STRATEGY AND PLANNING

Research subject matter	Research program to which subject matter is concerned

Research Subject: Analysis of transgenic expression.

Various discussions were held with both senior and junior staff members of EMBRAPA/CENARGEN biotechnology group regarding the analysis of transgenic expression. The strategies and experimental priorities used in the consultant's laboratories, for the qualitative, quantitative and distributional analysis of transgenic expression, were described and discussed in detail. Discussions were also undertaken to instigate further research collaborations and exchange of staff between EMBRAPA/CENARGEN and the consultant's university.

6.6 ACTIVITIES IN SUPPORT OF OTHER CENTERS AND UNIVERSITIES IMPROVING THE RESEARCH CENTERS LINKS WITH ABROAD

Subject matter on which links were recommended	Persons, centers and universities recomended for contact

In the consultant's opinion the biotechnology group has, because of its work, already established and excellent network of links with overseas research groups of the highest grade in their field of work.

6.7 PUBLICATIONS AND REPORTS UNDERTAKEN WITH THE CONSULTAT'S PARTICIPATION

Author(s)*	Title of publication or Report and other bibliografic identification

It is confidently anticipated that the research initiated during this consultancy will lead to various publications and reports from the CENARGEN/EMBRAPA biotechnology group. The first of these should be completed within two to three months of this consultancy.

* Personal, institutional, etc.

6.8 SUPPORT PROVIDED TO EMBRAPA RESEARCHERS IN THESIS AND DISSERTATION WORK

Name of the student	Thesis subject matter and sinthesis of advice

6.9 OTHER ACTIVITIES DEVELOPED BY THE CONSULTANT

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7. OTHER NATIONAL SYSTEM CENTERS, APART FROM DUTY STATION CENTER, ASSISTED BY THE CONSULTANT

Research center	Area of assistance provided by the consultant

8. CONSULTANT'S SUGGESTIONS AND TECHNICAL OR INSTITUTIONAL RECOMMENDATIONS FOR THE IMPROVEMENT OF THE RESEARCH SERVICE

General Background:

Now that a range of basic protocols for the production of some transgenic plants by genetic engineering are established (eg Schell, 1987; Kuhlemeier et al., 1987) , the biotechnology group at EMBRAPA/CENARGEN has quite correctly, in the consultant's opinion, identified the requirement to develop an "in-house" ability to undertake the detailed analysis of the "when and where" of transgenic expression. This capability is, of course, crucial to the successful application of genetic engineering techniques since there will frequently be a requirement for the positional and temporal transgenic expression to be highly regulated; there is, after all, little point to genetic engineering if the 'new' gene is expressed at the 'wrong' time or in the wrong place.

Some aspects of the highly regulated control of gene expression in plants have already been described, for example in monocot grains (eg Kreis et al, 1986) legume seeds (eg Boulter et al., 1987) and self-incompatibility mechanisms (eg Cornish et al., 1987). Other work has indicated that the control of gene expression may be primarily at the transcriptional level, probably within the non-coding flanking gene sequences, but with post-translational controls also operating to modify expression at the translational level (eg Croy et al., 1988). Cis acting elements that enhance gene expression in a tissue-specific and temporally regulated manner have been identified (eg Chen et al., 1988) ; as well as the nature of some of the precise sorting mechanisms by which plant proteins are then directed to their correct cellular locations (eg Della-Cioppa et al., 1987). It has also been demonstrated that such tissue-specific regulation (eg Colot et al., 1987; Croy et al., 1988) and protein target ing (eg Tague and Chrispeels, 1987) may be utilised in controlling transgenic expression.

The analysis of the fine control of transgenic expression can only be carried out by a combination of molecular biological and histo/cyto-chemical techniques (Kang et al., 1988). This consultancy has been concerned with the introduction and establishment of histochemical techniques in the EMBRAPA/CENARGEN group to enable it to monitor progress in work on transgenic expression. A number of different approaches are possible for assaying control of transcriptional and translational products. The histological assay of plant m RNAs by in situ hybridisation (eg Harris and Croy, 1986) is not, in the consultant's knowledge, being undertaken anywhere in Latin America; there are, in fact, only a few laboratories in the world where this type of work on plant tissues is currently being undertaken successfully. The assay of translational products is dependent upon the protein's properties; for example the expression of enzymes has frequently been used to monitor the success of genetic transfer. In one case the enzyme (GUS) can also be used for distributional analysis as well as for qualitative/quantitative assays. Although apparently straightforward, Jefferson et al.(1987) " mphasise that meaningful interpretation of histological analysis in terms of extent of chimeric gene activity, whether by in stu hybridisation methods or by histochemistry..... is not a trivial or straightforward matter". In an extension from such work they conclude , for example, that "it is no longer adequate to describe the 35S promoter as constitutive solely on the basis of expression in all plant organs, when there may be a strong dependence of transcription on cell type or cell cycle".

An alternative approach to the use of GUS, and that introduced to EMBRAPA/CENARGEN by the consultant, is the utilisation of immunological techniques for the assay of

gene expression. The advantage of such an approach is that it can be used for rapid screening and/or accurate quantitative analysis by ELISA and for distributional analysis by immunohisto- and cyto- chemical techniques (eg Kang et al., 1988). It has the additional merit of actually assaying the gene product of interest rather than another compound in a chimeric construct.

Techniques for localisation of specific proteins at optical and electron microscope levels are now established within EMBRAPA/CENARGEN and should allow for future 'in-house' assays of the detailed effects of, for example, regulatory sequences, targeting sequences etc in chimeric gene constructs used in preliminary work to identify and isolate those specific sequences required for construction of 'cassettes' to be used for controlled transgenic expression.

SUGGESTIONS AND RECOMMENDATIONS:

The application of immunocytochemical techniques should be only one part of the assay facilities avaible within the biotechnology group; they should be allied with the routine application of Western blotting and ELISA techniques.

The group should consider the potential versatility of the Gus system for studying control of transgenic expression. N t only can the tissue distribu tion of Gus activity be monitored by enzyme reaction and deposition of a blue stain product (x-glu is probably the best commercially available substrate at the moment) but the system can also be adapted to immunolocalisation of intracellular studies which are not possible with the chromogenic procedures. The consultant has left an aliquot of anti-gus primary antibody (raised in rabbit) with the other reagents donated to the programme.

Although the technique of in sity hybridisation is still being developed for application to plant material the EMBRAPA/CENARGEN group should follow advances with particular care. It is an immensely powerfull practical tool which is now being used to great effect in studies of the control of both normal and transgenic expression in plants, by groups such as those of Golberg, Clark e, Monsanto etc. It should, in due course, be introduced to the assay capabilities of the biotecnology group.

Organisational decisions will be required regarding (1) responsibility for assays of gene expression, and (2) priorities in the work of the personnel associated with the electron microscope unit.

In (1) the main option is to have either different individuals responsible for different facets of the assay procedures or a system in which one member of staff is responsible for all assaying associated with a particular project. The choice may be controlled by the technical versatility of staff members, but where this is not limiting, the second option may be more appropriate for a variety of both organisational and personnal reasons.

Regarding (2), it is often the case that a successful microscope unit generates numerous requests from within the larger department. Requests for work to be undertaken must be examined on a strict scientific basis; the case that "it would be nice to have a picture of...." must be given very low priority if the microscope unit is to be allowed to develop an integral part of the significant biological investigations of the overall research group. It may be inappropriate to have work requests directed straight to, for example, an EM technician; such request must be

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phytohemagglutinin, is transported to the vacuole in transgenic yeast J. Cell Biol. 105:1971-1979.

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9. AGREEMENTS OR COMMITMENTS ESTABLISHED WITH EMBRAPA RESEARCHERS IN-SERVICE OF THE FUTURE DEVELOPMENT OF RESEARCH IN THE CONSULTANT'S FIELD OF SPECIALIZATION

It was always anticipated that the work initiated during this consultancy would be continued in collaborations between EMBRAPA/CENARGEN and the university of Durham. Such collaborations should include continuation of specific research programmes, further in-service training of EMBRAPA staff both in Brasilia and in the consultant's laboratory, and the development of the more sophisticated assay systems (eg in sity hybridisation) as described above. Options for funding such collaborations are currently being investigated.

10. CONSULTANT'S COMMENTS ON CIRCUMSTANCES WHICH AFFECTED THE CONSULTANCY WORK

It is a pleasure to acknowledge the interest, help and assistance provide by Drs. de Castro, Sampaio and Gander and their staffs in the course of this consultancy. Although Brasilia was upset by industrial action, public holidays and storms the work continued every day and was only interupted by the infortunate failure of the Em (a problem all too common in labs all over the world, particulary it seems, at critical times).

06/05/88

Date:

Signature

APPENDIX

REAGENTS DONATED TO PROJECT

Colloidal Gold - Antibody Conjugates for Immunocytochemistry

	0,5 ml	of	Goat Anti Rabbit IgG + 10mm Gold
	0,5 ml	of	Goat Anti Rabbit IgG + 20mm Gold
	0,5 ml	of	Goat Anti Rabbit IgG + 5mm Gold
Also	0,5 ml	of	Protein a Conjugated to 10 mm Gold

Laboratory Consumables for Light and Electron Microscope

A wide range (including 3.100 specimen grids; 1000 embedding capsules).

Photographic Reagents

Kodak 70mm roll film for electron microscope
Ilford multigrade paper (resin coated for high quality prints)
Ilford multigrade filter kit

Embedding Resin

1000 ml LR white resin

Silver Intensification Kit For Optical Microscope Immunocytochemistry

Tanssen Intense II Kit.

To Total Value US$ 1.500

GUIDE FOR ELLABORATION OF CONSULTANT'S FINAL REPORT

The first page of the Final Report is filled up by the Coordination of IICA/EMBRAPA Contract for a clear identification of consultancy and program.

From second page on there are two alternatives, either one should be selected by the Consultant, according with his/her best estimation of the type of report to submit.

One alternative (using the remainder pages) is for specialists engaged more with direct research, as well as other activities like advice in programming, evaluation and future needs for EMBRAPA's research programs.

The other alternative is better adapted to consultancies in institution strengthening, with the following structure:

. Aknoledgments (optional)
. Summary (optional)
6 . Institutions assisted
7 . Cooperating staff (name and function)
8 . Persons interviewed for the development of the activities (name and function)
9 . Activities developed (based on duties and responsibilities listed in the job description)
10. Results of the activities (includes analysis and evaluation)
11. Conclusions
12. Suggestions and recommendations (to meet te objectives of the project)
13. Occurence of facts outside IICA that affected the performance of consultancy activities.
14. List of literature cited or having relevance to the work in the area of consultancy (optional)
15. Agencies referred to in the text of having relevance to the work in the area of consultancy (optional)
16. Signature and date

Obs.: PLEASE SEND THE ORIGINAL AND ONE COPY TO THE IICA/EMBRAPA CONTRACT COORDINATOR, CAIXA POSTAL 09-1070, 71.600 BRASILIA, DF, BRAZIL.

Programa II. Geração e Transferência de Tecnologia

O Programa de Geração e Transferência de Tecnologia é a resposta do IICA a dois aspectos fundamentais: (i) o reconhecimento, por parte dos países e da comunidade técnico-financeira internacional, da importância da tecnologia para o desenvolvimento produtivo do setor agropecuário; (ii) a convicção generalizada de que, para aproveitar plenamente o potencial da ciência e da tecnologia, é necessário que existam infra-estruturas institucionais capazes de desenvolver as respostas tecnológicas adequadas às condições específicas de cada país, bem como um lineamento de políticas que promova e possibilite que tais infra-estruturas sejam incorporadas aos processos produtivos.

Nesse contexto, o Programa II visa a promover e apoiar as ações dos Estados membros destinadas a aprimorar a configuração de suas políticas tecnológicas, fortalecer a organização e administração de seus sistemas de geração e transferência de tecnologia e facilitar a transferência tecnológica internacional. Desse modo será possível fazer melhor aproveitamento de todos os recursos disponíveis e uma contribuição mais eficiente e efetiva para a solução dos problemas tecnológicos da produção agropecuária, num âmbito de igualdade na distribuição dos benefícios e de conservação dos recursos naturais.

INSTITUTO INTERAMERICANO DE COOPERAÇÃO PARA A AGRICULTURA

O Instituto Interamericano de Cooperação para a Agricultura (IICA) é o organismo especializado em agricultura do Sistema Interamericano. Suas origens datam de 7 outubro de 1942, quando o Conselho Diretor da União Pan-Americana aprovou a criação do Instituto Interamericano de Ciências Agrícolas.

Fundado como uma instituição de pesquisa agronômica e de ensino; de pós-graduação para os trópicos, o IICA, respondendo às mudanças e novas necessidades do Hemisfério, converteu-se progressivamente em um organismo de cooperação técnica e fortalecimento institucional no campo da agropecuária. Essas transformações foram reconhecidas oficialmente com a ratificação, em 8 de dezembro de 1980, de uma nova convenção, que estabeleceu como fins do IICA estimular, promover e apoiar os laços de cooperação entre seus 31 Estados membros para a obtenção do desenvolvimento agrícola e do bem-estar rural.

Com um mandato amplo e flexível e com uma estrutura que permite a participação direta dos Estados membros na Junta Interamericana de Agricultura e em seu Comitê Executivo, o IICA conta com ampla presença geográfica em todos os países membros para responder a suas necessidades de cooperação técnica.

As contribuições dos Estados membros e as relações que o IICA mantém com 12 Países Observadores, e com vários organismos internacionais, lhe permitem canalizar importantes recursos humanos e financeiros em prol do desenvolvimento agrícola do Hemisfério.

O Plano de Médio Prazo 1987-1991, documento normativo que assinala as prioridades dc Instituto, enfatiza ações voltadas para a reativação do setor agropecuário como elemento central do crescimento econômico. Em vista disso, o Instituto atribui especial importância ao apoio e promoção de ações tendentes à modernização tecnológica do campo e ao fortalecimento dos processos de integração regional e sub-regional.

Para alcançar tais objetivos o IICA concentra suas atividades em cinco áreas fundamentais, a saber: Análise e Planejamento da Política Agrária; Geração e Transferência de Tecnologia; Organização e Administração para o Desenvolvimento Rural; Comercialização e Agroindústria, e Saúde Animal e Sanidade Vegetal.

Essas áreas de ação expressam, simultaneamente, as necessidades e prioridades determinadas pelos próprios Estados membros e o âmbito de trabalho em que o IICA concentra seus esforços e sua capacidade técnica, tanto sob o ponto de vista de seus recursos humanos e financeiros, como de sua relação com outros organismos internacionais.

Esta publicação foi reproduzida na Gráfica do Escritório do IICA no Brasil, em Brasília, em abril de 1988, numa tiragem de 200 exemplares.

Responsáveis pela reprodução: Jadir José dos Santos e Murillo Sodré da Silva.

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CA/EMBRAPA


IICA
PM-A4/BR-88-11
Autor
Título Immunocytochemical localizatio
of transgenic problems in
plant tissues
Fecha Devolución
Nombre del solicitante

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