New Methods and Results in Monitoring Field Release of Genetically Modified Organisms

J. Schiemann[1], H. Backhaus[1], U. Commandeur[1], A. Dietz-Pfeilstetter[1], B. Engelen[1], F. Gebhard[1], H. Heuer[1], R. Koenig[1], D.-E. Lesemann[1], E. Maiß[2], A. Matzk[1], F. Niepold[1], K. Smalla[1] and R. Casper[1]

Abstract

In Germany, biosafety research accompanying field releases of genetically modified organisms is mainly supported by the Federal Ministry of Education, Science, Research and Technology (BMBF). The project "Biosafety research accompanying field releases of transgenic plants", performed at the Federal Research Centre for Agriculture and Forestry, consists of three main topics:

- Transcapsidation and recombination of viruses in transgenic plants expressing virus genes,
- Gene transfer from transgenic plants to other organisms,
- Influence of foreign gene expression in transgenic plants on plant-associated microorganisms.

Besides giving results from the BMBF-supported biosafety research, we are reporting on molecular investigations on the outcrossing of herbicide tolerance genes in the frame of a project accompanying field releases of BASTA-tolerant oilseed rape. We are also reporting on a joint action (in which more than 15 German laboratories are involved) to develop polymerase chain reaction (PCR)-based techniques for the detection of transgenes/GMOs in potato, yoghurt, and raw sausage.

In the frame of this biosafety research, different methods for the sensitive detection of GMOs have been developed which will be useful for post-release and post-commercialization monitoring of GMOs.

Introduction

Genetically modified organisms (GMOs), as well as non-GMOs, are neither inherently risky nor inherently safe; whether or not the release and commercialization of an organism pose a risk to human health or the environment depends on the characteristics of the organism, and the circumstances of its application. In the context of the "Precautionary Principle", the relative lack of familiarity with GMOs justifies appro-

1 Federal Biological Research Centre for Agriculture and Forestry, Institute for Biochemistry and Plant Virology, Messeweg 11/12, 38104 Braunschweig, Germany

2 University of Hannover, Institute for Plant Diseases and Plant Protection, Herrenhäuser Str. 2, 30419 Hannover, Germany

priate risk assessment for their application. The risk assessment is focused on organisms and traits which are of commercial and social importance. In the frame of these studies - including field work - monitoring methods for the identification of GMOs have been developed.

The field releases accompanied by biosafety research include the following transgenic plants:

- Rhizomania-resistant sugarbeet containing the Rhizomania virus coat protein gene and additional marker genes; field releases performed by Planta GmbH, Einbeck, in Lower Saxony and Bavaria.
- Potato plants with the yeast invertase gene and potato plants with an antisense sequence of the granule-bound starch synthase gene; field releases performed in Lower Saxony by the Institut für Genbiologische Forschung Berlin GmbH (IGF).
- Potato plants expressing T4-lysozyme supplied by the Bundesanstalt für Züchtungsforschung an Kulturpflanzen (BAZ) in Quedlinburg have been investigated in the greenhouse.

A. Transcapsidation and recombination of viruses in transgenic plants expressing virus genes

Attempts to detect heteroencapsidations and viral gene recombinations in transgenic sugarbeet plants expressing the coat protein gene of beet necrotic yellow vein virus; comparison of heterologous encapsidations in mixed infections and in transgenic plants; determination of the natural variance of different plum pox potyvirus isolates in comparison to possible recombination events of the virus in transgenic plants

Introduction

Coat protein-mediated and other forms of pathogen-derived resistances have proved to be among the most promising approaches for the protection of plants against virus infections. However, some unintended side effects, such as heteroencapsidations (Lecoq *et al.*, 1993; Maiss *et al.*, 1995a) and viral genome recombinations (Greene and Allison, 1994) have raised concerns that this form of resistance may present a biological risk (e.g. de Zoeten, 1991). The first plants with genetically engineered virus resistance to be released in small field trials in Germany were transgenic sugarbeet plants expressing the coat protein gene of beet necrotic yellow vein virus (BNYVV). In the past years we have investigated whether heteroencapsidations or recombinations can be observed in such plants.

In simultaneous studies with *Nicotiana benthamiana* expressing the coat protein gene of plum pox potyvirus (PPV), heterologous encapsidations in transgenic plants and in mixed infections were compared. In addition, the natural variance of different plum pox potyvirus isolates in comparison to possible recombination events of the virus in transgenic plants was determined.

Methods and results

In attempts to detect heteroencapsidations in transgenic sugarbeet plants expressing the coat protein gene of BNYVV, we have used immunoelectron microscopical decoration tests (Maiss *et al.*, 1995a) and immunocapture reverse transcription (RT) PCR (Koenig *et al.*, 1995a). By means of immunoelectron microscopy and immunocapture RT PCR we were unable to obtain evidence that the coat protein of BNYVV can encapsidate the genomes of rod-shaped, filamentous or isometrical sugarbeet viruses, i.e. of beet soil-borne, tobacco mosaic, beet mosaic, beet yellows, beet mild yellowing or tobacco necrosis viruses. In the studies described below this method was found to be very successful for the detection of heteroencapsidations among potyviruses (Maiss *et al.*, 1995a). By means of immunocapture RT-PCR we were also unable to detect the BNYVV coat protein gene in the capsids of the other sugarbeet viruses (Koenig *et al.*, 1995b). This fact suggests that heteroencapsidations or recombinations - if they occur at all in transgenic sugarbeet plants expressing the coat protein gene of BNYVV - are apparently rare events.

Recombinations are most likely to occur between different strains of the same virus. Using molecular techniques we have recently shown the existence of two major strain groups of BNYVV. While in most countries the A-type occurs, the B-type is prevalent in Germany and France (Kruse *et al.*, 1994; Koenig *et al.*, 1995a). The transgenic sugarbeet plants express nucleotides 1 to 827 of RNA 2 of the A-type of BNYVV. In order to find out whether in plants infected with B-type BNYVV a recombination between the sequences from the two virus strains takes place, we have designed PCR primers which encompass the region of nucleotides 448 to 955 of BNYVV RNA 2. PCR products obtained from recombinant virus would contain the A-type sequence in their 5'-half and the B-type sequence in their 3'-half. With an artificial gene construct we could show that PCR products from virus with a recombinant genome would be readily detectable by means of SSCP or RFLP analyses, even in the presence of a considerable excess of PCR products from virus with a non-recombinant genome. In a limited number of experiments we have so far obtained no evidence that a recombination between transgene-derived A-type and virus-derived B-type sequences occurs in transgenic sugarbeets which express the BNYVV coat protein gene.

Polyclonal antisera for PPV detection were produced by immunizing rabbits with purified virus particles or purified PPV coat protein expressed in *E. coli*. In addition, a mouse monoclonal antibody specific for PVY was used to determine the heterologous encapsidation in mixed infections with PPV. For visualizing particles with mixed coat proteins, immunoelectron microscopy with gold-labelled goat anti-rabbit or goat anti-mouse antibodies as second antibodies was used. The size of the gold particles was 5 nm for the anti-rabbit antibodies and 15 nm for the anti-mouse antibodies which allowed the simultaneous detection of PPV and PVY coat proteins in a virus particle.

The coat protein genes of different plum pox potyvirus (PPV) isolates were PCR-amplified after trapping of virus particles with PPV-specific antibodies. The cDNA fragments were subsequently cloned and sequenced. A comparison was made with

additional published PPV coat protein sequences, to evaluate the baseline of natural variance within the coat protein genes.

Heterologous encapsidation has been observed in mixed infections of PPV and potato virus Y (PVY) and in transgenic *Nicotiana benthamiana* plants (Maiss *et al.*, 1995a). Although heterologous encapsidations occur with a relatively high frequency within the genus potyvirus, preliminary aphid transmission experiments of heterologous encapsidated PPV isolate NAT from transgenic plants failed. The labelling technique, using polyclonal and monoclonal antibodies in immunosorbent electron microscopy, is sensitive to detect heterologous encapsidated virus particles. It should be possible to adapt this technique to other transgenic plant/virus combinations for the detection of heterologous encapsidations. The only prerequisite is the availability of specific antibodies.

Comparison of the coat proteins of different PPV isolates revealed a considerable degree of variance of PPV isolates from different geographic origins, suggesting a relatively high divergence of isolates (Deborré *et al.*, 1995; Maiss *et al.*, 1995b). The phylogenetic relationship of coat proteins was established using the complete coat protein sequences of 24 PPV isolates available. The phylogenetic tree reveals a cluster of M-type isolates, consisting of a Bulgarian isolate (PPV-BUL), of the PS and o6 isolate from Yugoslavia, the SK68 isolate from Hungary and the Cambridge Gage isolate from Germany. Within the D-type cluster one will find a panel of other isolates from Germany, France and Yugoslavia. A subcluster within the D-type isolates consists of isolates which have a deletion in the coat protein gene. The highest divergence to all other isolates was shown by the El-Amar isolate from Egypt (Fig. 1).

The use of virus-specific antisera and virus-specific oligonucleotides allows a rapid and reliable amplification of coat protein genes. This technique makes it possible to investigate the naturally occurring variance in virus isolates.

B. Gene transfer from transgenic plants to other organisms

Genetic and molecular analysis of hybrids between sugarbeet and related beet varieties

Introduction

A possible scenario when releasing transgenic virus- and herbicide-resistant sugarbeet might be the emergence of more compatible weed beets due to the transfer of the resistance genes to other *Beta vulgaris* varieties (Newbury *et al.*, 1989). Reciprocal gene exchange between cultivated sugarbeet and wild beets in seed production areas was demonstrated by Santoni and Bervillé (1992) and is probably responsible for the occurrence of weed beets in production fields (Boudry *et al.*, 1993).

Expression of transgenes is regulated by endogenous as well as exogenous determinants, among them the genetic constitution of a plant. Therefore, the expression of

newly introduced genes is not predictable after transfer to another genetic background. Also, it was reported that transgenes can be lost or poorly transmitted to subsequent generations (Spencer *et al.*, 1992). Investigations on the characteristics of transgenes after transfer to different genetic backgrounds are therefore of importance for evaluating the consequences of such gene transfer events.

Methods and results

Based on the assumption that gene transfer from transgenic sugarbeet to related *Beta vulgaris* varieties is possible, the objective of our project was the analysis of the resulting hybrids with respect to transgene expression and inheritance. Using two different transgenic sugarbeet lines (L3 and L4) for pollination of mangel plants (*Beta vulgaris* ssp. *vulgaris*) and of two wild beet accessions (*Beta vulgaris* ssp. *maritima*), five F1 hybrid lines were obtained. Of these, four were subject to vernalization and selfing. Fig. 2 shows an outline of the overall experimental design.

Expression analysis revealed that leaf as well as root expression of the BNYVV coat protein in transgenic hybrids is in the same range as in the original transgenic sugarbeet lines. About 7% of the mangel x L4 hybrids (3 out of 43), however, were found to express the coat protein at a very low level in leaves. Root expression was on the average about 10 fold lower than leaf expression, if equal amounts of total protein were used in the enzyme-linked immunosorbent assay (ELISA). All transgenic hybrids did express the Basta® resistance (*bar*) gene. Mild symptoms of herbicide damage (necroses) after Basta® dot application appearing in some of the transgenic wild beet hybrids were not caused by the active ingredient D,L-phosphinothricin (D,L-PPT). Spraying with 5 l/ha Basta® was tolerated by all of the transgenic hybrids whereas nontransgenic beet plants were killed or severely damaged.

The inheritance ratios after selfing of F1 hybrids revealed Mendelian inheritance for mangel x L3 and wild beet x L4 hybrids while mangel x L4 hybrids did not show the expected 3:1 ratio but a 2:1 (31:16) ratio of transgenic versus non-transgenic plants. A possible reason for this deviation might be a non-tolerable homozygous state for the integration event of L4 in the genetic background of the mangel hybrids.

Investigation on horizontal gene transfer from transgenic sugarbeet plants into plant associated microorganisms and soil microorganisms

Introduction

Bacterial antibiotic or herbicide resistance genes are still frequently used as selectable markers for transgenic plants. Due to well-known problems caused by antibiotic-resistant pathogens, concerns arose about the large scale use of transgenic plants containing antibiotic resistance genes. Therefore, horizontal transfer of such resistance genes from plants to microorganisms has often been discussed as potential unwanted effect of transgenic plants on the soil microbiota. However, there is no clear evidence for gene transfer from plants to microorganisms to date.

Transgenic plant DNA can be released into the environment, e.g., from senescent or rotting plant material. The persistence of free DNA released in soil is of great importance with respect to potential gene transfer by transformation. If the released free transgenic plant DNA is immediately degraded by extracellular nucleases, transgenic DNA is unlikely to be taken up by competent soil bacteria. On the other hand, long-term persistence of DNA might enhance the likelihood of transformation-like processes to occur.

The most probable mechanism for gene transfer from plants to microorganisms is natural transformation requiring the uptake of free DNA by naturally competent soil bacteria and the integration of the foreign DNA into the bacterial genome (Stewart, 1989). In order to detect gene transfer from plants to microorganisms under field conditions there must not only be mechanisms to allow uptake and replication in the new host but, perhaps most importantly, a selection for the host expressing a new trait. Even though competent soil bacteria are known, only a few reports showed that natural transformation occurs in non-sterile soils (Lorenz *et al.*, 1992; Gallori *et al.*, 1994).

Methods and results

Persistence of transgenic plant DNA in soil was investigated for transgenic tobacco (*aac*C1, Paget *et al.*, 1993), transgenic petunia (NOS-*npt*II, Becker *et al.*, 1994) and transgenic sugarbeet (*bar*/TR1, TR2/*npt*II, 35S/BNYVV-*cp*, Smalla *et al.*, 1995). The persistence of the construct in soil was tracked by direct DNA extraction from soil followed by PCR-based amplification of the construct. Appropriate primer selection allowed an unequivocal detection of the transgenic construct besides the naturally occurring genes. With this methodology the presence of the construct could be detected but no information was gained on its localization inside rotting plant material, as free DNA adsorbed to soil surface, in metabolically active, dormant, or dead cells. The sensitivity for detection of the construct in total soil DNA extracts should be determined since it will vary with the soil type, the lysis and purification protocol and PCR conditions. Limits of detection were determined for the construct used in transgenic sugarbeet with three different primer sets (*bar*/TR1; TR2/*npt*II, 35S-BNYVV-*cp*) to be around 10^2

target sequences per gram soil (Gebhard *et al.*, unpublished). Transgenic sugarbeet-DNA was detectable in soil samples taken from a disposal site 6, 12 and 18 months after transgenic sugarbeet had been ploughed into soil. Although for soil sampled 6 months after shredding and incorporation of transgenic sugarbeets into the soil, PCR products were obtained with all 3 primer sets, no PCR products were detected in DNA extracted from soils sampled 12 or 18 months after incorporation using the 35S/cp primer system.

Detection of horizontal gene transfer events can be performed by analyzing bacteria after a preliminary cultivation step. In order to obtain information on the presence of the construct in non-culturable bacteria, the bacterial fraction recovered directly from soil can be analyzed for the transgenic DNA. However, screening of cultivated bacteria for the presence of the construct has the advantage that putative transconjugants can be further characterized. Provided that the transferred resistance genes are expressed in their new hosts, selective cultivation can be applied. In a project accompanying the field release of Rhizomania-resistant transgenic sugarbeet containing the *npt*II and the *bar* gene under the control of the TR1/TR2 promoter and the BNYVV coat protein gene under the control of the 35S promoter, soil bacteria were screened for horizontal gene transfer events. Since the level of expression of the *npt*II gene in different soil bacteria was unknown, resuspended soil samples were plated onto nutrient media containing 100 µg/ml, 10 µg/ml or no kanamycin. Approximately 3,000 soil bacteria isolated from different samplings were randomly picked and analyzed by cell hybridization using the construct as probe. Several positive colonies giving slightly positive signals were further confirmed by PCR. None of the colonies contained the construct. To improve the sensitivity of the detection, total DNA was extracted from bacterial lawns growing at the lowest dilution, analyzed by PCR and hybridization. Until now the construct was never detectable in the fraction of bacteria grown on plate count agar. To obtain information on gene transfer into the non-culturable fraction of soil bacteria DNA, the bacterial fraction was recovered from soil samples by different blending and centrifugation steps. DNA extracted from the bacterial fraction was analyzed by PCR. However, positive PCR signals are difficult to interpret since it is almost impossible even after several DNase treatment steps to ensure the absence of free DNA. Especially in soils with a high content of clay particles a complete separation of bacteria from clay particles is difficult, and DNA adsorbed to clay minerals will resist DNase attacks to a certain extent.

Recently developed molecular approaches allow a sensitive and specific tracking of transgenic DNA in soil. However, detection of gene transfer into bacteria not accessible to cultivation still remains complicated. Although transgenic plant DNA is detectable in soil for a longer period, horizontal gene transfer from plant to bacteria was not detectable under laboratory and field conditions until now. The present results of the investigation focused on horizontal gene transfer from plants to microorganisms allow to conclude that such transfer events, in case they occur at all, will be rare. Furthermore, the ongoing environmental release of *npt*II-containing bacteria with sewage or

manure slurries into agricultural soils did not result in a dissemination of the *npt*II gene amongst soil bacteria. This fact might serve as an argument to ease concerns about the possible impediment of medical therapies through the use of the *npt*II gene in transgenic plants.

Detection of persisting transgenic *Agrobacterium tumefaciens* in transgenic potato or sugarbeet plants tested in field trials

Introduction

The potato and sugarbeet plants tested in the field have been transformed by *Agrobacterium*-mediated gene transfer. The foreign genes in genetically modified agrobacteria persisting in transgenic plants could be transferred into other bacteria in the environment, particularly in the rhizosphere depending on the host range of the plasmids used for transformation. Plasmids can be exchanged between cells of different species or even different genera (Bradbury, 1986). There are also possible routes for the transfer of agrobacteria from plant to plant, e.g., by phloem-feeding insects such as whiteflies (Zeidan and Czosnek, 1994), and by physical spread over vegetative tissues and through soil (Mogilner *et al.*, 1993). This could cause an undesirable spread of transgenes in the ecosystem. Therefore, it would be judicious to eliminate agrobacteria before releasing transgenic plants into the field. The possibility of transmitting genetically engineered agrobacteria into seed progeny, as well as vegetative propagules such as tubers, also needs to be evaluated adequately. The detection of agrobacteria requires sensitive and reliable methods.

Methods and results

Different techniques were developed to detect *Agrobacterium tumefaciens* persisting in transgenic plants. Cultivation-based methods take advantage of selectable antibiotic resistance markers. The chromosomally encoded rifampicin resistance of the *Agrobacterium* strains used for plant transformation turned out to be extremely useful for the selection of persisting agrobacteria after a selective enrichment step due to low bacterial background with a rifampicin resistance phenotype. Selective enrichment of *Agrobacterium* from macerated plant material is highly sensitive and appropriate for routine testing of large numbers of samples. Bacteria obtained after selective cultivation can be identified as agrobacteria by microbiological standard techniques and immunological methods and screened for the presence of the construct used for transformation by dot blot cell hybridization. Dot blot hybridization-positive samples can be verified by PCR using primer systems specific for the construct to exclude hybridization signals due to naturally occurring *npt*II or *bar* sequences. Identification of dot blot and PCR-positive samples with the BIOLOG and *Agrobacterium*-specific polyclonal antibodies can be used to detect persisting transgenic agrobacteria besides potential transconjugants. However, it cannot be excluded that bacteria affected by

environmental stress resist cultivation by enrichment.

An alternative for detecting non-culturable agrobacteria is the total DNA extraction from plant material combining cell homogenization using liquid nitrogen with enzymatic and alkaline-lysis, followed by PCR amplification using primer systems specific for the transgenic *Agrobacterium tumefaciens*. The PCR-based approach supplies information on the presence of a certain sequence in the sample only but not on the localization of the detected sequence, i.e. in culturable, non-culturable, or dead bacteria, or in extracellular DNA. Furthermore, the limit of detection is impeded by the unfavorable ratio of plant DNA to bacterial DNA.

Further methods developed to detect agrobacteria in plants (Matzk *et al.*, 1996) include tissue-print immunoblotting, scanning, and transmission electron microscopy. These methods are less sensitive than the previously mentioned detection techniques, and more appropriate for investigations on the localization of agrobacteria in the different parts of the plant than for monitoring large numbers of transgenic plants for persisting transgenic agrobacteria.

Leaf material sampled randomly from transgenic and non-transgenic sugarbeet and potato plants at the release site was macerated and enriched in nutrient broth supplemented with rifampicin and cycloheximide (to prevent fungal growth). Enrichment cultures, inoculated with a defined number of agrobacteria in a background of plant material or without, showed that there was no inhibition of the growth of agrobacteria caused by the plant material. Even cultures inoculated with a single agrobacterium cell and incubated in a background with 0.1 g of tobacco tissue could be enriched to the late exponential phase after 3 days. When no agrobacteria were inoculated the OD_{600} did not increase. The enrichment broth was plated onto selective agar containing rifampicin and cycloheximide. Colonies with similar morphology as *Agrobacterium tumefaciens* were tested by slide agglutination test using a polyclonal antiserum, ketolactose test, and BIOLOG. None of the tested rifampicin-resistant bacteria were identified as *Agrobacterium*. Furthermore, all the colonies obtained from transgenic plant material after selective enrichment were screened with dot blot hybridization for the presence of the construct used for transformation and for the *vir*G gene specific for *Agrobacterium*. No positive hybridization signals except for the positive controls were observed. Transgenic *Agrobacterium* DNA could not be amplified from total DNAs extracted from the above mentioned leaf material by PCR. There are no indications for the persistence of transgenic *Agrobacterium tumefaciens* in transgenic sugarbeet and potato plants grown under field conditions.

C. Influence of foreign gene expression in transgenic plants on plant-associated microorganisms

Influence of foreign gene expression in transgenic potatoes on plant-associated microorganisms; impact of genetically modified organisms or xenobiotics on the activity and composition of the microflora

Introduction

Several attempts to genetically engineer crop plants for resistance to pathogenic microorganisms are currently in progress (Strittmatter and Wegener, 1993). Strategies which do not target a specific pathogen might influence other microbes. Subsequently, the soil as a sink and source for plant-colonizing bacteria might be changed in its microbial community structure. As bacteria are important, e.g. for soil fertility or plant growth promotion, the detection of induced changes in community structures is essential to risk assessment.

Most experimental approaches for analyzing the composition or diversity of microbial communities rely on characterizing constituents. These methods are time-consuming because of basic problems in the identification of microbes which are often morphologically uniform. Monitoring effects on the microflora in field releases by these techniques is not feasible because of the complexity of soil and plant-associated communities. Forthis purpose "community level" methods which allow the comparison by reproducible "fingerprints" are preferable.

Methods and results

In our work two techniques were evaluated to produce comparative fingerprints for monitoring treatment effects. One approach - the "metabolic fingerprinting" - is based on the potential of communities to utilize sole-carbon-sources (Garland and Mills, 1991), the other - the "genetic fingerprinting" - makes use of the diversity of ribosomal genes (Muyzer *et al.*, 1993). By differences in fingerprints, shifts in the bacterial communities can be detected and subsequently analyzed in detail to determine the affected populations.

Both "community level" methods were compared to established methods and investigated regarding reproducibility of the fingerprints, and sensitivity to detect bacterial community shifts. The methods were applied to monitor (1) for expected effects of a pesticide treatment on the bacterial community of an agricultural soil, and (2) for effects of T4-lysozyme expressed and secreted by transgenic potato plants on bacterial communities in the rhizo- and phyllosphere.

1) The "metabolic fingerprint" using BIOLOG microtiterplates

The wells of the commercially available BIOLOG microtiterplates (BIOLOG Inc., Hayward, CA) contain 95 different carbon sources in a dried form and a redox dye

which takes a color when inoculated microorganisms oxidize the substrate. Bacteria were extracted from plant surfaces or soil and the cell suspensions were incubated in the microtiterplates. The color development was quantified and normalized patterns were compared between communities using principal component analysis and a multivariate statistical test for significant differences.

The BIOLOG patterns of microbial communities from potato rhizosphere and phyllosphere were largely different. Typical carbon sources were utilized depending on the habitat. Patterns were reproducible and the methodological error was significantly smaller than the variability between replicate plants. The sensitivity of the method was high enough to detect significant differences between the phyllosphere communities of two potato varieties which utilized the same carbon sources but at slightly differing rates.

In the phyllosphere studies, some evidence was found that fungi and gram-positive bacteria may not contribute efficiently to the patterns. Thus a restriction of this monitoring method to fast-growing heterotrophic aerobic gram-negative bacteria must be taken into account when conclusions are drawn from results.

2) The "genetic fingerprint" by Temperature Gradient Gel Electrophoresis (TGGE)

Due to the large amount of non-culturable bacteria within the microbial populations a community is not sufficiently characterizable by traditional cultivation methods. TGGE allows a community analysis at the DNA level by separating PCR products that have the same length but different sequences. During the electrophoresis the electric field is overlapped by a temperature gradient that causes melting of the nucleic acids. If the fragments reach their melting point, they slow down their migration in the electric field which results in distinct bands in the gel. Since this technique is actually developed to recover point mutations it is highly sensitive. It is obvious that this technique is suitable to characterize a community by molecules of phylogenetic significance, like the 16S ribosomal $\sqrt{\text{RNA}}$ genes.

A microbial population can be analyzed by extracting nucleic acids from a habitat, amplifying the mentioned DNA-region by PCR using phylogenetically conserved sequences as primers, and separating the PCR-products by TGGE. The resulting pattern is the "genetic fingerprint" of the community.

Reproducible patterns were found for soil, potato rhizosphere, and phyllosphere. The latter shows much less complexity and higher variability.

By serial dilution of the DNA from extracted rhizosphere communities and subsequent PCR-TGGE, it was shown that the TGGE patterns represent the most abundant species whereas rare community members (<1%) hardly contribute to the patterns. Attempts to analyze less abundant but functionally important bacterial groups using group-specific primers are currently in progress.

3) Application of the presented fingerprinting methods

A pesticide treatment with an expected impact on microbial soil communities was

used as a model to compare traditional community analysis to the presented fingerprinting methods. In this experiment three different pesticides were applied to soil samples which were incubated for several weeks.

As in pesticide registration procedures (Anderson, 1990) the metabolic parameters such as dehydrogenase activity, nitrogen turnover, glucose-induced respiration, and long-term respiration were monitored at different times after application. The data were statistically analyzed to observe the impact of the different treatments on the communities.

The investigated shifts in the community structure were confirmed by the fingerprinting methods. The statistical analysis of BIOLOG patterns showed in all observations the same significant differences from the control as the glucose-induced respiration measurements.

The TGGE pattern of the investigated replicates was highly reproducible. Two weeks after pesticide application the patterns of the differently treated communities began to differentiate. The patterns obtained by the pesticide with the most detectable effect by all methods were dominated by five species representing bands eight weeks after application. A detailed pattern analysis by sequencing these dominant bands is still in progress. The specific identification of the affected community members opens the opportunity to interpret the ecological impact of the investigated treatments.

A second application of the community-level methods was the monitoring for effects of genetically modified potato plants on the associated bacterial communities. The plants produce T4-lysozyme for protection against the pathogen *Erwinia carotovora*. As the lysozyme does not specifically target this bacterium the community structure might be changed.

The investigations in the greenhouse preceding a field experiment did not give evidence for considerable community shifts. In the BIOLOG approach, the carbon sources utilized by phyllosphere or rhizosphere communities from transgenic and control plants were the same, and no significant differences could be found in the patterns of metabolic rates. Also the TGGE-patterns of the rhizosphere communities were very similar, and for the phyllospheres the variability between replicate plants was too high to detect any differences (Fig. 3).

These findings were consistent with the results from characterization and classification of bacterial strains isolated from the plant surfaces: Only minor shifts in the relative abundance of enteric bacteria, *Pseudomonas, Arthrobacter* and *Bacillus* could be found. The finding that the number of gram-positive bacteria was reduced on leaves of the transgenic potatoes at the end of the growth season has to be confirmed.

However, the bacterial species composition found on plants grown in the greenhouse and in the field differed considerably, indicating that the results obtained in the greenhouse cannot be extrapolated to field experiments. Additionally, the variability of the phyllospheres of replicate plants was much higher in the greenhouse which decreased the sensitivity to detect effects of the T4-lysozyme. Thus, further investiga-

tions accompanying a field experiment have been started.

Investigations on the possible formation of a new reservoir of pathogens during cultivation of transgenic potatoes

Introduction

The aim of the investigations was to determine whether there is a change in the susceptibility of the two transgenic potato lines "anti GBSS" and "Invertase" in comparison to the original, non-transgenic variety "Désirée" by collecting potato pathogenic bacteria and fungi, like *Phytophthora infestans* and *Erwinia carotovora*. The polymerase chain reaction (PCR) was applied to allow the identification of very slight differences in the reaction of the potato pathogenic microorganisms. In addition, a biotest with the fungus *Phytophthora infestans* was conducted to compare possible changes of the biological reaction of the two transgenic potato lines in comparison to the non-transgenic variety "Désirée".

Methods and results

For the PCR-mediated detection of *Phytophthora infestans*, the causal agent of late blight disease of potato, a set of primers was derived from a satellite DNA being in tandem repeat and occurring about 15 times per haploid genome, generating a 362 bp DNA fragment as an amplificate. Using a repeat sequence as a primer target, the sensitivity of the detection increased. A PCR detection of the bacterium *Erwinia carotovora*, the causal agent of soft rot disease, was performed with primers which had already been published. The primers were derived from pathogenicity-associated genes representing the pectine lyase gene complex. To detect as small quantities as possible of plant material infested by fungi and bacteria, the extraction procedures and PCR conditions were optimized, resulting in a further increase of the threshold of the detection limit and also in useful instructions for a PCR detection of the two potato pathogenic microorganisms.

The four different races of *Phytophthora infestans* which occur in Germany could be detected in potato tissues at a concentration corresponding to 100 ng of freeze-dried mycelium. Experiments for the determination of the detection threshold of artificially inoculated potato tissues revealed that only 6 zoospores were necessary to obtain a visible PCR signal on an agarose gel when no recombinant potato plants were used (Niepold and Schöber, 1995).

To investigate the application of the PCR for the detection of *Erwinia carotovora*, twelve non-recombinant *in vitro* potato plantlets were randomly chosen. All of them looked healthy and none of them exhibited any disease symptoms. However, after PCR only two of them could be identified as being free from the bacterium. Since the detection threshold of the PCR was determined to be about 10 cfu per ml using artificially inoculated potato leaves, latent bacteria were detectable in non-transgenic

plantlets at the same concentration level (Niepold, 1994).

The PCR approach was used to investigate the possibility of detecting higher susceptibilities of the transgenic potatoes in comparison to the non-transgenic "Désirée". Therefore, field samples were collected during the vegetative period and, as an example, *Phytophthora infestans* was surveyed by PCR using the primers developed. As a result, no measurable increase of susceptibility of the transgenic potato lines compared to the non-transgenic potatoes could be detected.

When using *Phytophthora infestans* in a biotest, a slight difference in the behavior of the two different transgenic potato lines "anti GBSS" and "Invertase" was observed. A tendency towards a slightly increased susceptibility of the transgenic potato lineswas recognized. On the other hand, this variation could have also been generated by the somaclonal variation of the two transgenic potato lines.

D. Detection of herbicide tolerance genes

Development of fast and simple assay procedures to detect herbicide resistant oilseed rape plants

Introduction

Most of the applications for transgenic plants to be released into the field or placed on the European market refer to herbicide-tolerant plants. The problem of identification of the transgenic plants, the detection of outcrossing evidences as well as monitoring of survivability, establishment ability, and dissemination capacity of the transgenic plants requires the development of sensitive and reliable screening methods for the routine detection of the transgene. Resistance against the herbicide Basta® can be obtained after transformation using the *pat* (phosphinothricin acetyltransferase) gene (Wohlleben *et al.*, 1988).

Methods and results

Oilseed rape transformed by *Agrobacterium*-mediated gene transfer was obtained from AgrEvo, (Frankfurt/Main, Germany). Plants expressing the *pat* gene do not show growth inhibition after application of the herbicide (5 l/ha). To verify this screening result, DNA was extracted from leaf tissues using a fast procedure protocol (Gebhardt *et al.*, 1989), followed by dot blot hybridization using a specific probe for the *pat* gene. All dot blot-positive samples can be confirmed by PCR with one primer (CAGAACTCGCCGTAAAGACT) in the CaMV 35S promoter region and the other primer (CAACTCCCACAACACCGACC) in the coding region of the *pat* gene. The resulting 734-bp fragment confirmed the identification of the transgene. The expression of the *pat* gene was determined by the ELISA technique using a polyclonal antibody raised in goat (obtained from AgrEvo). The sensitivity and specificity of this technique were increased by modifying the following aspects: direct binding of the pro-

tein to the plate, preincubation of the specific antibody in the sap of untransformed oilseed rape leaves, and use of a secondary antibody (rabbit anti-goat alkaline phosphatase-labelled).

These methods are routine methods for the detection of the *pat* gene at the molecular and protein level, allowing the screening of a large number of samples in a short time. We are using the described methods for investigations on the outcrossing of herbicide tolerance genes in the frame of a project accompanying field releases of BASTA-tolerant oilseed rape. Trap plants around the isolation distance of 200 m and randomly taken samples from the 6 m border row will be analyzed for the presence of the *pat* gene and its expression.

E. Detection of transgenes / GMOs in foodstuffs

Joint action to develop PCR-based techniques for the detection of transgenes / GMOs in potato, raw sausage, and yoghurt

Introduction

Also in Germany there are activities to develop methods for the detection of foods produced by genetic engineering. The foods under evaluation consist of the GMO itself and food produced by using genetically modified microorganisms. Detection methods based on PCR/hybridization procedures which have been developed for potato and raw sausage (containing genetically modified starter cultures), are currently being developed for yoghurt, and planned for tomato (ketchup) and soybean.

Methods and results

To develop and evaluate a standardized protocol, ring tests are being performed which are coordinated by the Federal Institute for Health Protection of Consumers and Veterinary Medicine (BgVV). For the development of a PCR/hybridization procedure, a transgenic potato line containing the yeast invertase and the hygromycine phosphotransferase (*hpt*) marker gene was chosen. Seventeen laboratories representing Government Authorities, Authorities of several German countries (Bundesländer), university institutes, and private institutions performed the analysis according to a standard protocol containing a detailed description of DNA preparation, gel electrophoresis, PCR, and Southern analysis, including equipment and chemicals. Ten coded samples per laboratory, including 3 to 5 transgenic tubers, as well as positive and negative control tubers were provided together with the PCR primers and the hybridization probe. With the PCR primers

5' CGC CGA TGG TTT CTA CAA 3'

5' GGC GTC GGT TTC CAC TAT 3'

an 839 base pair sequence of *hpt* gene was amplified and hybridized with a probe of the following sequence: 5' GGC CCA TTC GGA CCG CAA GGA ATC G 3'.

In the ring test, 163 samples were correctly identified. Three transgenic samples gave false negative results. One non-transgenic sample was false positive. The false positive and one false negative samples were presumably exchanged. On the basis of these results, the protocol became a part of the official compilation of methods for foodstuff analyses according to the German food law.

Acknowledgement

This project is partially supported by the Federal Ministry of Education, Science, Research and Technology (Project number 0310582A).

References

1) Anderson, J.P.E., Casle, D., Ehle, H. Eichler, D., Laermann, H.-T., Maas, G. and Malkomes, H.-P. (1990): Richtlinien für die amtliche Prüfung von Pflanzenschutzmitteln, Teil VI, 1-1 (2. Aufl.): Auswirkungen auf die Aktivität der Bodenmikroflora. Braunschweig: Biologische Bundesanstalt für Land- und Forstwirtschaft.
2) Becker, J., Siegert, H., Logemann, J. and Schell, J. (1994): Begleitende Sicherheitsforschung zur Freisetzung gentechnisch veränderter Petunien. *In:* Bundesministerium für Forschung und Technologie (ed.), Biologische Sicherheit. Forschung Biotechnologie. Bd. 3 (1994): 563-578.
3) Boudry, P., Mörchen, M., Saumitou-Laprade, P., Vernet, Ph. and van Dijk (1993): The origin and evolution of weed beets: consequences for the breeding and release of herbicide resistant transgenic sugarbeets. Theor. Appl. Genet. **87** : 471-478.
4) Bradbury, J.F. (1986): Guide to plant pathogen bacteria. Pp. 6-15, CAB International, Slough, UK.
5) De Zoeten, G. (1991). Risk assessment: do we let history repeat itself ? Phytopathology **81** : 585-586.
6) Deborré, G., Maiss, E. and Jelkmann, W. (1995): Biological and molecular biological investigations of several plum pox potyvirus (PPV) isolates. Acta Horticulturae **386** : 253-262.
7) Gallori, E., Bazzicalupo, M., Canto, L.D., Fani, R., Nannipieri, P., Vettori, C. and Stotzky, G. (1994): Transformation of *Bacillus subtilis* by DNA bound on clay in non-sterile soil. FEMS Microbiology Ecology **15** : 119-126.
8) Garland, J.L. and Mills, A.L. (1991): Classification and characterization of heterotrophic microbial communities on the basis of patterns of community-level sole-carbon-source-utilization. Appl. Environ. Microbiol. **57** : 2351-2359
9) Gebhardt, C., Ritter, E, Debener, T., Schachtschabel, U., Walkemeier, B, Uhrig, H. and Salamini, S. (1989): RFLP-analysis and linkage mapping in *Solanum tuberosum*. Theor. Appl. Genet. **78** : 65-75.
10) Greene, A.E. and Allison, A.F. (1994): Recombination between viral RNA and transgenic plant transcripts. Science **263** : 1423-1425.

11) Koenig, R., Lesemann, D.-E. and Maiss, E. (1995b): Attempts to detect heteroencapsidations or other non-intended side effects in transgenic sugarbeet expressing the coat protein gene of beet necrotic vellow vein virus (BNYVV). Mitt. Biol. Bundesanst. Land- und Forstwirtsch., Berlin Dahlem, 309, 31-39. Key Biosafety Aspects of Genetically Modified Organisms, J. Landsmann and R. Casper (eds)

12) Koenig, R., Lüddecke, P. and Haeberlé, A.M. (1995a) : Detection of beet necrotic yellow vein virus strains, variants and mixed infections by examining single-strand conformation polymorphisms of immunocapture RT-PCR products. J. Gen. Virol. **76** : 2051-2055.

13) Kruse, M., Koenig, R., Hoffmann, A., Kaufmann, A., Commandeur, U., Solovyev, A.G., Savenkov, I. and Burgermeister, W. (1994): RFLP analysis of RT-PCR products reveals the existence of two major strain groups of beet necrotic yellow vein virus. J. Gen. Virol. **75** : 1835-1842.

14) Lecoq, H., Ravelonandro, M., Wipf-Scheibel, C., Monsion, M., Raccah, B. and Dunez, J. (1993): Aphid transmission of a non-aphid-transmissible strain of zucchini yellow mosaic potyvirus from transgenic plants expressing the capsid protein of plum pox potyvirus. Molecular Plant-Microbe Interactions **6** : 403-406.

15) Lorenz, M.G., Reipschläger, K. and Wackernagel, W. (1992): Plasmid transformation of naturally competent *Acinetobacter calcoaceticus* in non-sterile soil extract and groundwater. Archives of Microbiology **157** : 355-360.

16) Maiss, E., Deborré, G., Jelkmann, W. and Casper, R. (1995b): Complete nucleotide sequence of a plum pox potyvirus isolate (PPV-SC) deriving from sour cherries and influence of a coat protein sequence motif on aphid. Acta Horticulturae **386** : 340-345.

17) Maiss, E., Koenig, R. and Lesemann, D.-E. (1995a): Heterologous encapsidation of viruses in transgenic plants and in mixed infections. *In:* D.D.Jowes (ed.), Third International Symposium on the Biosafety Results of Field Tests of Genetically Modified Plants and Microorganisms. Monterey, California, Nov. 13-16, 1994: 129-139.

18) Matzk, A., Mantell, S. and Schiemann, J. (1996): Localization of persisting agrobacteria in transgenic tobacco plants. Molecular Plant-Microbe Interactions **9** (5) : 373-381.

19) Mogilner, N., Zutra, D., Gafny, R. and Bar-Joseph, M. (1993): The persistence of engineered *Agrobacterium tumefaciens* in agroinfected plants. Molecular Plant-Microbe Interactions **6** : 673-675.

20) Muyzer, G., De Waal, E.C. and Uitterlinden, A.G. (1993): Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microbiol. **59** : 695-700

21) Newbury, J., Todd, G., Godwin, I. and Ford-Lloyd, B. (1989): Designer beet - the impact of genetic engineering on sugarbeet. Beet Review **57** : 41-45.

22) Niepold, F. and Schöber-Butin, B. (1995): Application of the PCR technique to detect *Phytophthora infestans* in potato tubers and leaves. Microbiological Research

150 : 379-385.

23) Niepold, F. (1994): Anwendung der Polymerase-Kettenreaktion (PCR) zum Nachweis von *Erwinia carotovora* ssp. *atroseptica*, dem Erreger der Schwarzbeinigkeit und Naßfäule bei Kartoffeln. Nachrichtenbl. Deut. Pflanzenschutzd. **46**(11) : 243-246.

24) Paget, E. and Simonet, P. (1993) : Evidence of gene transfer in soil via transformation. *In*: Commission of the European Communities (ed.), BRIDGE/BIOTECH. Final sectorial meeting on biosafety and first sectorial meeting on microbial ecology, October 24-27, 1993, Granada, Spain, p. 32.

25) Santoni, S. and Bervillé, A. (1992) : Evidence for gene exchange between sugarbeet (*Beta vulgaris* L.) and wild beets: consequences for transgenic sugarbeets. Plant Mol. Biol. **20** : 578 - 580.

26) Smalla, K., Gebhard, F., Van Elsas, J.D., Matzk, A. and Schiemann, J. (1994): Bacterial communities influenced by transgenic plants. *In:* D.D. Jones (ed.), Third International Symposium on The Biosafety Results of Field Tests of Genetically Modified Plants and Microorganisms, November 13-16, 1994, Monterey, USA, pp 157-167.

27) Spencer, T.M., O'Brien, J.V., Start, W.G. and Adams, T.R. (1992): Segregation of transgenes in maize. Plant Mol. Biol. **18** : 201-210.

28) Stewart, G.J. (1989): The mechanism of natural transformation. *In:* S.B. Levy, R.V. Miller (eds), Gene transfer in the environment. McGraw-Hill Publishing Company, New York, 1989, 139-164.

29) Strittmatter, G. and Wegener, D. (1993): Genetic engineering of disease and pest resistance in plants: present state of the art. Z. Naturforsch. 48c: 673-688.

30) Wohlleben W., Arnold, W., Broer, I., Hillemann, D., Strauch, E. and Pühler, A. (1988): Nucleotide sequence of the *phosphinothricin-N-acetyltransferase* gene from *Streptomyces viridochromogenes* Tü494 and its expression in *Nicotiana tabacum*. Gene **70** : 25-37.

This publication is dedicated by Joachim Schiemann to Professor Dr. Benno Parthier, his esteemed academic teacher and supervisor of his thesis in Halle in the mid-1970s.

Professor Parthier, Director of the Institute for Plant Biochemistry, Halle, and President of the "Deutsche Akademie der Naturforscher Leopoldina", celebrates his 65th anniversary on 21 August 1997.

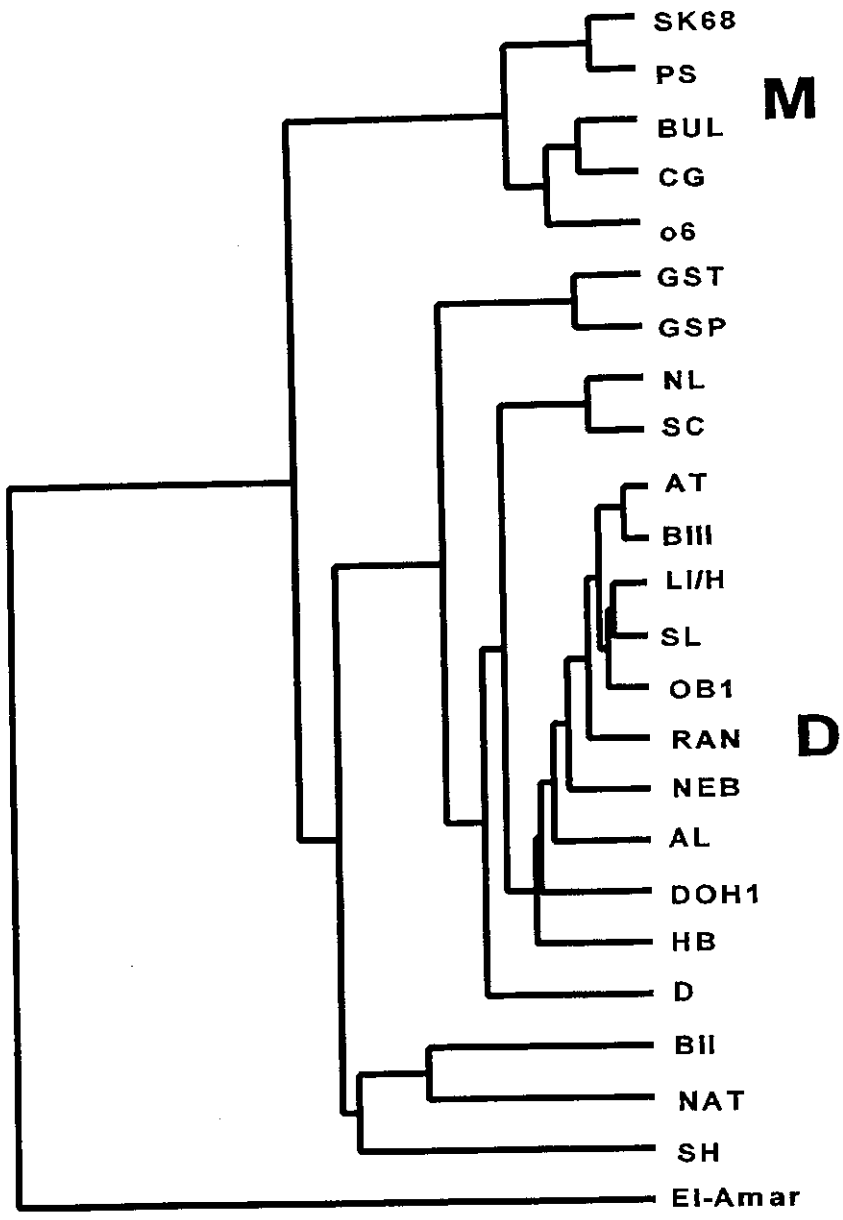

Fig. 1 Phylogenetic tree of coat proteins of different plum pox potyvirus isolates (Maiss *et al.*, 1995b; Deborré *et al.*, 1995)

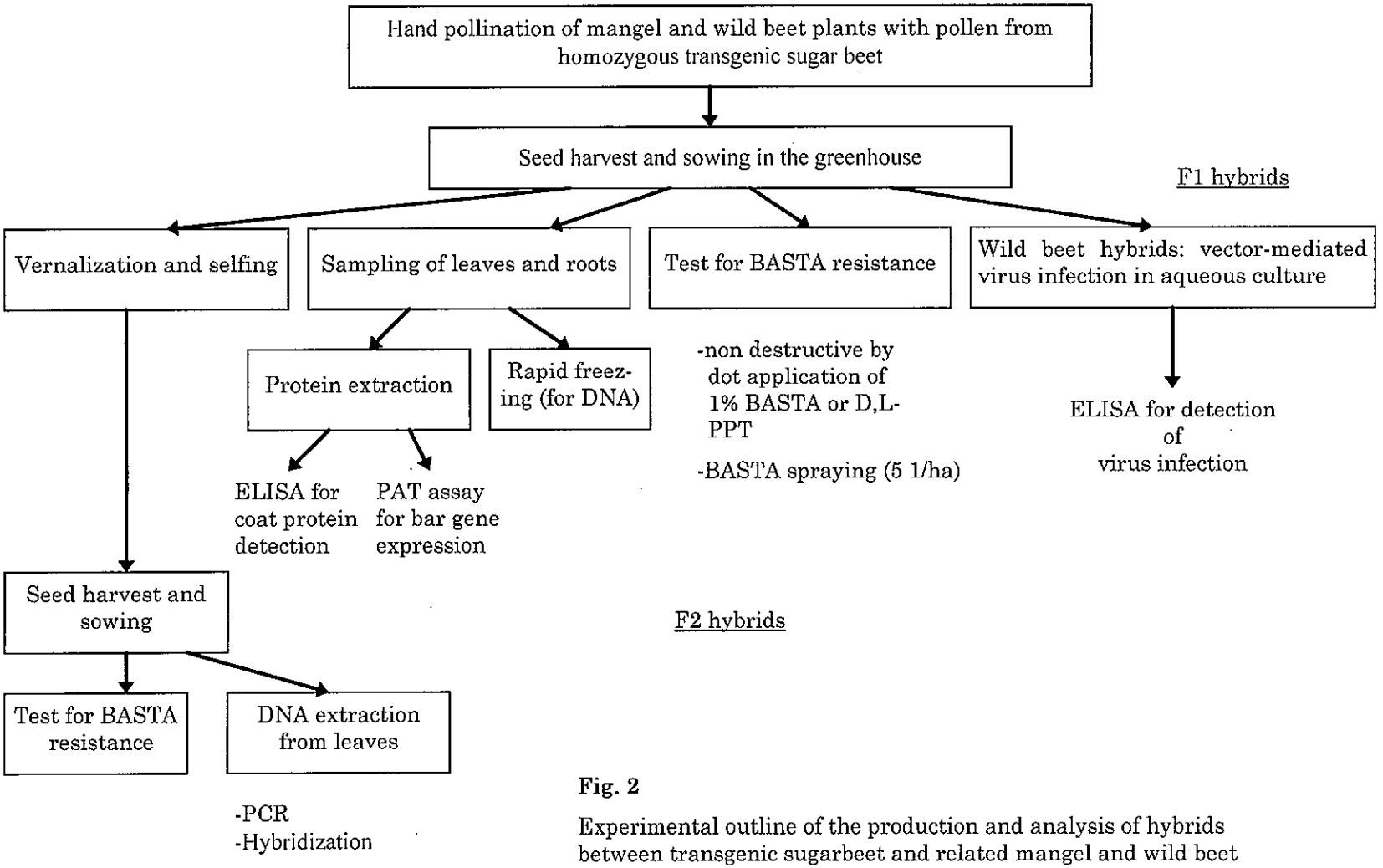

Fig. 2

Experimental outline of the production and analysis of hybrids between transgenic sugarbeet and related mangel and wild beet plants

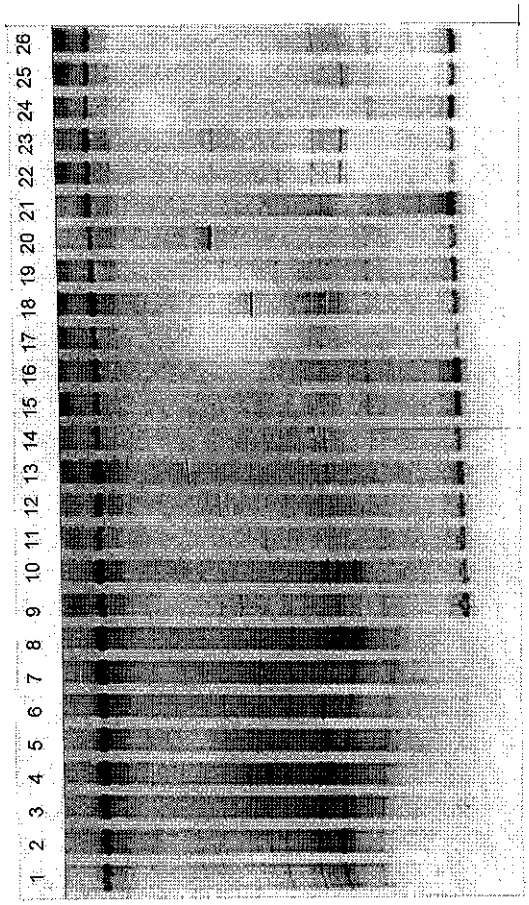

Fig. 3 Sequence specific separation by TGGE of PCR-derived 16S ribosomal gene fragments from bacterial communities extracted from rhizospheres (lanes 1-8) or leaves (lanes 9-26) of potato plants: Comparison of transgenic potatoes (lanes 5-8 and 18-26) with non-transgenic potato plants (lanes 1-4 and 9-17)