SESSION 3

GENETIC MANIPULATION AND CROP PROTECTION

CHAIRMAN PROFESSOR P. R. DAY

SESSION ORGANISER DR R. B. FLAVELL

POSTER PAPERS	3A-1 to 3A-5
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POTATO PLANTS TRANSFORMED WITH T-DNA FROM *A. TUMEFACIENS*

M.H. BURRELL, D. TWELL, J. ROBERTS and G. COMS

Biochemistry Department, Rothamsted Experimental Station, Harpenden, Herts., AL5 2JQ.

ABSTRACT

Potato plants transformed with T-DNA from a shoot-inducing *A. tumefaciens* strain were isolated and analysed for plant development and T-DNA structure and transcription. Early in development the transformed cells formed stunted and frequently branching shoots without roots that were grown in culture. Repeated grafting of the shoots onto rootstocks of normal potato or tomato rootstocks and subsequent growth in soil, induced a more normal morphology and development. The plants, however, tuberised strongly. Upon vernalization transformed tubers sprouted and the newly developing plants formed roots and second generation tubers. Associated with the normalisation in phenotype was a reduction in the steady state concentration of most, if not all, hormone related T-DNA coded RNA's.

INTRODUCTION

Agrobacterium tumefaciens is a common soil bacterium which is unique in that it induces tumours on plants after infection of wounded cells. Its host range is limited to dicotyledonous plants. A tumour develops when a bacterial DNA fragment is transferred from a bacterial plasmid, a Ti or tumour inducing plasmid, into the nuclear genome of a plant cell. This transferred DNA (T-DNA) is expressed in the transformed cell as RNA and protein and the effects of the expression products lead to abnormalities in plant hormone metabolism causing tumerous growth. In some cases the tumerous state can be partially overcome. Many aspects of the cellular and molecular biology of the transformation process itself. The structure and function of various T-DNA's and characteristics of transformed cells and plants have been summarised and discussed in a number of recent reviews (Binns 1984, Hooykaas and Schilperoort 1983, Nester *et al.* 1984).

The poster illustrates that it is possible to regenerate transformed potato plants from tumour cells. These transformed plants can form tubers and flowers and the tubers will sprout to give second generation transformed plants.

RESULTS AND DISCUSSION

A shoot inducing strain of *Agrobacterium tumefaciens* (LBA 1501) was used to induce a tumour on the stem of the potato shoot of c.v. Maris Bard. Three months after infection a tumour with shoots had formed. The tumour was grown in culture and shoots were either transformed and showed stunted growth or untransformed and mostly normal (Coms *et al.* 1983). Transformed shoots were grafted onto rootstocks of untransformed shoots and after a short period in culture potted up. Their morphology gradually became more normal. Following a second grafting their morphology became even more normal although frequent side shoots and aerial tubers developed. When the graft was kept in the soil, below ground tubers formed which after a period of vernalisation sprouted and formed roots. Despite

slightly abnormal plant development, apparently normal second generation tubers were formed (Ooms and Lenton, subm.).

The structure of the T-DNA and its transcription into mRNA in the transformed potato line was studied after size fractionation on agarose gels by electrophoresis blotting and hybridisation. Of the eight transcripts previously detected in tobacco callus (Willmitzer et al. 1982) five were identified. The abundance of at least three of the transcripts decreased in the grafted plants and tubers compared with the shoot cultures (Burrell et al. subm.).

CONCLUSIONS

The regeneration of derivatives of Solanum tuberosum cv. Maris Bard transformed with T-DNA from A. tumefaciens, showed that whole plant genetic manipulation of potato cultivars is possible. Besides the demonstration of this principle, the ease with which Ti-T-DNA transformed potato plants can be isolated and the observed strong tendency to tuberise may turn out to be of considerable scientific importance. Gene expression in tubers of any gene inserted into shoot inducing T-DNA and cointroduced into potato can be studied soon in such strongly tuberising plants. Perhaps of even greater significance is that the results demonstrated that T-DNA genes themselves can be used to bring about specific changes in growth and development of potato. This opens up the novel prospect, hitherto impossible because of technical reasons, to study the expression of specific (modified) genes important in controlling growth and development in potato and their influence on the agronomically important process of tuberisation.

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THE PRACTICAL APPLICATION OF COMPLEMENTARY DNA PROBES TO VIRUS DETECTION IN A POTATO BREEDING PROGRAMME

R.E. BOULTON, G.J. JELLIS, D.C. BAULCOMBE, A.M. SQUIRE

Plant Breeding Institute, Maris Lane, Trumpington, Cambridge CB2 2LQ, U.K.

ABSTRACT

Potato clones in large-scale breeding programmes are conventionally assessed for resistance to infection with potato virus Y (PVY) and potato leafroll virus (PLRV) by growing on tubers from plants exposed to the viruses and scoring visual symptoms in the second year. This is time-consuming and can be inaccurate. Resistance to potato virus X (PVX) is assessed by scoring visual symptoms following challenge with the virus by grafting.

Sap spot hybridisation (SASH) using ^{32}P-labelled complementary DNA probes was compared with ELISA in experiments to establish a reliable method of PVY and PLRV detection, and hence assessment of resistance, in the winter following virus exposure.

Results suggest that a winter test is feasible by either detection method, but that SASH is more sensitive than ELISA for PLRV, at least under certain conditions. SASH was also more sensitive for PVX when symptomless plants from graft tests were checked for presence of virus.

SASH is easy and rapid, but the preparation of radioactive probes imposes some limitation on its general availability at present.

INTRODUCTION

The most damaging potato viruses occurring in the UK are potato virus Y (PVY) and potato leafroll virus (PLRV). These are aphid-transmitted and are widespread in ware growing districts, making the production of healthy home-saved seed tubers difficult (Jellis & Boulton, 1984). Virus infection can also cause losses, sometimes severe, in the high-grade seed producing districts, due to downgrading of crops at inspection. The mild mosaic viruses, including potato virus X (PVX), are also potentially damaging.

Varieties with high resistance to virus infection can significantly reduce losses. At the Plant Breeding Institute, potato clones in the breeding programme are assessed for virus resistance at an early stage in their development as potential varieties. Assessment for PVY and PLRV involves exposing plants of each clone to viruliferous aphid vectors in replicated field plots. Since infected plants frequently do not show symptoms in the year of primary infection the conventional procedure has been to plant out tubers of exposed plants in the following year and to visually assess the enhanced symptom expression in the resultant secondarily-infected plants. This procedure involves two growing seasons and uses a large amount of land. Also there is evidence from Wooster (1984) and from our own work (unpublished) that some potato genotypes show very mild or no symptoms although they are susceptible to infection, thus complicating visual assessment of resistance. Selection based on a reliable test for the presence of virus carried out in the winter following virus exposure would drastically reduce the numbers of tubers retained for the second year's planting. Only tubers from the most resistant clones would be regrown for

further assessment. Hypersensitive reaction to PVX is determined by grafting a scion of a PVX-infected plant onto the plant under test. Resistant plants show an extreme hypersensitive reaction. Non-resistant plants may develop mosaic symptoms or may remain symptomless; the latter are retested to determine whether or not graft transmission has been successful.

The use of ELISA (enzyme-linked immunosorbent assay) for the detection of plant viruses (Clark & Adams 1977) has vastly improved the speed and sensitivity of assaying large numbers of samples. Recently, nucleic acid hybridisation using ^{32}P-labelled complementary DNA (cDNA) probes has been used to detect viroids (Owens & Diener, 1981) and viruses (Maule *et al*. 1983, Harrison *et al*. 1983). This technique, called sap spot hybridisation (SASH), has also been developed at the Plant Breeding Institute for the detection of potato viruses (Baulcombe *et al*. 1984a, 1984b) and provides an alternative method of assaying for PVY, PLRV and PVX.

This report presents some results of investigations to establish procedures for reliably testing large numbers of potato clones, using ELISA and SASH.

MATERIALS AND METHODS

For the PVY and PLRV tests in 1983, clones produced in a somaclonal breeding programme and exposed to viruliferous aphids in the previous year, were grown in pots in a glasshouse without supplementary lighting (Jellis *et al*. 1984). Leaf samples were taken 10 weeks after emergence.

For the PVY and PLRV tests in 1984, tubers from plants exposed to the viruses in replicated field plots were harvested in September 1983. After storage at 10-15°c in the dark, tubers were exposed to Rindite in January to ensure uniform dormancy break. Five weeks later eyepieces from the sprout end of each tuber were planted in pots in a glasshouse with supplementary lighting. Leaf samples from the resultant plants were taken after 5 weeks growth. For the PVX tests, leaf samples were taken from a range of genotypes challenged with virus by grafting with infected scions.

Sap was extracted from leaves by roller press and frozen at -20°C until required. The same crude sap samples used for SASH were used in the preparation of sub-samples for ELISA.

The method of Clark & Adams (1977) was used for ELISA. Antisera specific to PVY or PVX was prepared at the Plant Breeding Institute; commercially prepared antiserum (Inotech Diagnostik AG) was used for the PLRV tests. For ELISA tests crude sap was diluted 1:9 in phosphate buffered saline + 0.05% Tween and 2% polyvinylpyrrolidone. Absorbances were read on a Titertek Multiskan colorimeter.

The preparation, labelling, hybridisation and autoradiography procedures for cDNA probes specific to each virus were essentially as described previously by Baulcombe *et al*. (1984b) for PVX. Sample application was carried out by spotting 2 µl of each crude sap sample onto nitrocellulose filters, which had been wetted in water followed by wetting in 3 M NaCl, 0.3 M Na citrate (20 x SSC). The filters were then air dried and baked *in vacuo* at 80°C for two hours. The developed autoradiographs were scored visually, using a light box.

TABLE 1

Detection of PVY, PLRV and PVX in potato plants by ELISA and SASH

Virus	Year	Number of plants			
		ELISA + SASH +	ELISA - SASH -	ELISA - SASH +	ELISA + SASH -
PVY	1983	19	53	4	0
	1984	164	101	2	1
PLRV	1983	18	41	30	1
	1984	306	38	7	7
PVX	1983	28	50	21	0

+ = virus infected, - = virus free, as determined by each technique

RESULTS

The results for PVY and PLRV in 1983 and 1984, and for PVX in 1983, are presented in Table 1.

The results for PVY show good agreement between the two methods in both years (98% agreement over the two years).

For PLRV in 1983, 30 plants (33%) were SASH positive but ELISA negative. Immunosorbent electron microscopy (Derrick, 1973) on sap from random samples of these plants showed that they contained leafroll virus particles. No particles were found in the sample which was SASH negative but ELISA positive. All other samples which were ELISA positive were also SASH positive. In 1984 there was 96% agreement between the two methods.

In the 1984 tests, both methods detected many virus-free plants amongst those genotypes which were most likely to have virus resistance i.e. with at least one resistant parent.

For PVX, all plants which were ELISA positive were SASH positive; an additional 22% were SASH positive but ELISA negative.

DISCUSSION

Both methods appear to have the same degree of sensitivity for detection of PVY. For PLRV, SASH appears to be more sensitive, at least under certain conditions. The plants tested for PLRV in 1983 were grown in warm and somewhat shaded conditions, and PLRV concentration may have been lower in many of these plants than in those tested in 1984. SASH was also more sensitive than ELISA for PVX detection.

Further work (unpublished) has indicated that tests for PVY and PLRV on leaf sap are generally more reliable, by both SASH and ELISA, than tests on tuber sap.

Assessment of material from breeding programmes necessarily involves testing a large number of plants of differing genotypes, and this diversity

can affect the distribution and concentration of virus, and thus the ease of detection. The results presented in this report suggest that winter testing for PVY and PLRV after primary infection is a reliable method of detecting viruses and hence of assessing resistance. The sap spot hybridisation technique offers sensitivity of detection at least as good as ELISA. It has the distinct advantage that only 1-2 µl of untreated sap are needed for each test so sample preparation is easy and rapid. Two hundred samples can be placed on duplicate filters in one hour, and results can be obtained in 3 days. Furthermore, SASH results are unambiguously positive or negative, whereas sap from different plant genotypes can show wide variation in non-virus-specific reaction to antisera, sometimes making the interpretation of colorimeter readings difficult. Also, cDNA probes can show a very high degree of virus strain-specificity (Baulcombe et al. 1984b). The main disadvantage of the technique is that at present the use of radioactive probes requires preparation and processing by specialised laboratories, thus limiting its general availability. However, the development of non-radioactive probes is in progress.

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NUCLEIC ACID HYBRIDISATION TECHNIQUES FOR THE DETECTION OF PLANT PATHOGENS IN INSECT VECTORS

M.I. BOULTON, P.G. MARKHAM, J.W. DAVIES

John Innes Institute, Colney Lane, Norwich. U.K.

ABSTRACT

Existing spot hybridisation methods have been modified to give a rapid, sensitive technique suitable for the screening of plant pathogens in their insect vectors. Insects squashed in the presence of alkali onto nitrocellulose filters were hybridised with radioactive DNA probes; the response was linear with regard to DNA concentration. For maize streak virus (MSV) in its leafhopper vector the limit of detection was 2.5pg DNA or 12.5 pg virus per insect. Several spiroplasmas could also be detected in leafhoppers, some with greater reliability than by microscopical examination of the haemolymph. Application of the technique to acquisition studies for MSV characterised a time course for acquisition and identified some important features of virus/vector relationships in different leafhoppers.

INTRODUCTION

Many diseases of economically important crops are spread by insect vectors; these include diseases caused by viruses and spiroplasmas. Until recently the bioassay techniques used for detecting these plant pathogens in their insect vectors were insensitive or time consuming. However, some of the problems have been overcome with the routine use of two methods dependent upon immunological detection of the pathogens' coat protein or membrane proteins; ELISA, enzyme linked immunosorbent assay, and ISEM, immunosorbent electron microscopy.

A sensitive technique used in virus research for the detection of virus diseases in plant or animal tissues, spot hybridisation, relies upon the detection of the viral RNA or DNA immobilised on a solid phase. The basis of the assay is the formation of double stranded molecules between the nucleic acid under test and a complementary nucleic acid, which is radioactively labelled. The method has been found to be more sensitive than the immunological techniques for detection of viruses in plant sap extracts (Maule et al,1983). A recent report (Jayasena et al,1984) has suggested that a similar technique based upon hybridisation in a liquid phase is sufficiently sensitive to use for the determination of pathogen concentration in single aphid vectors.

In this paper we report the extension of this technique to the detection and assay of the DNA-containing geminivirus MSV; and three spiroplasmas, BC3, Spiroplasma citri and corn stunt spiroplasma, in leafhoppers. The technique has also provided information on the acquisition and transmission of MSV by various leafhopper species.

MATERIALS AND METHODS

MSV purification

The Nigerian isolate of MSV (a gift from Dr. Rossel, IITA), was purified from maize,(Zea mays) either according to the method of Bock et al.(1974) or a modification of this method using sodium citrate buffer pH 4.5, or sodium acetate buffer pH 4.5 for homogenisation.

Plant and Insect material.

Insects for the assay of MSV were given access to maize plants showing good virus symptoms. Access periods ranged from 3 hours to 12 days, all material being maintained at 29°C with a 14 hour photoperiod. The species tested were Cicadulina mbila, Cicadulina triangula, Cicadulina chinai, Dalbulus maidis, Circulifer tenellus and Macrosteles sexnotatus.

For spiroplasma assay, leafhoppers were infected either by intrahaemocoelic injection (Markham and Townsend, 1979) or by access to infected plants. Groups of 100-200 Euscelidius variegatus were injected with either S.citri (strain SPA-MD), CSS (Rio Grande Type, strain J2), or BC3, and maintained on ryegrass (Lolium multiflorum). C. tenellus were injected with S. citri and maintained on sugar beet (cv. Western Giant). D. maidis was maintained on maize, and was infected by feeding on CSS-infected maize plants.

Spot hybridisation

For Cicadulina spp. single leafhoppers were squashed directly onto nitrocellulose filters with the addition of 2.5ul of 0.5N NaOH. A flat-ended glass rod was found to be suitable. For E. variegatus 5ul was used. Standard DNA solutions, diluted in 0.5N NaOH, were spotted onto the filters in the presence of healthy insects in order to quantitate the viral DNA concentration. All samples were air dried and the filter then neutralised (Brandsma and Miller 1980). After drying, filters were agitated for 5 minutes in two changes of chloroform before being baked in a vacuum oven at 80°C.

Filter hybridisation

Nick translation of spiroplasma DNA was carried out following described methods (Rigby et al, 1977). ^{32}P-labelled cDNA was prepared to the single stranded MSV DNA by a modification of the random priming method (Taylor et al, 1976), using the Klenow fragment of DNA polymerase I (6U/ug MSV DNA) in place of reverse transcriptase. In later experiments a full length clone of the virus DNA was nick translated for use as a probe. Hybridisation and autoradiography were carried out as described by Maule et al, (1983). For quantification, areas of the filter containing single insects were excised and scintillation counted.

RESULTS

Using the spot hybridisation technique described (Materials and Methods) preparations of MSV DNA could be detected to a lower limit of 2.5pg/spot with a short autoradiographic exposure time (up to 48 hours). Alkali denaturation, theoretically not necessary for the single stranded DNA of MSV, increased the sensitivity of the method, particularly when insect material was also present.

To establish that the assay was quantitative, known amounts of MSV DNA were added to squashed leafhoppers on the filter and the filter processed

as described. The result showed a linear increase in radioactive counts with increasing amounts of viral DNA. Surprisingly, the presence of the squashed insect failed to affect the sensitivity of detection, although it was found necessary to take care to wash away excess probe non-specifically bound after hybridisation. The chloroform washing step as outlined in the Materials and Methods was also found to be critical; insufficient washing prevented the removal of background radioactivity after hybridisation. 1% SDS added to the 0.5M NaOH with the squashed insect did not obviate the chloroform washing step and failed to enhance the sensitivity of the method.

An alternative experimental approach where leafhoppers were homogenised in alkali, centrifuged and the supernatant spotted onto nitrocellulose, gave results equivalent to the "squash" method, but was a more laborious technique.

For all the leafhoppers tested, controls which had not been given access to the pathogen under test, always gave hybridisation signals not significantly above background.

Acquisition of MSV

Following a 7 day acquisition access period on MSV infected maize all 60 C.mbila tested were found to contain high, but variable, levels of MSV. Concentrations of MSV DNA were between 19pg and 1.6ng with a mean of 287pg for male and 382pg for female leafhoppers. However if duration of acquisition access was less than 4 days the mean virus concentration was higher in males than in females. These results probably reflect differences in behavioural characteristics between the two sexes.

Concentration of virus acquired by C.mbila increases with acquisition access time up to a maximum around 5 days. Virus was detected in 55% of the insects after a 3 hour access period. After 6 hours access feeding all C.mbila tested were positive although it was necessary to use prolonged autoradiography (greater than 48 hours).

When C.mbila was given a 7 day acquisition period on MSV-infected maize and then transferred to Zea perennis, which is resistant to MSV (Nault et al, 1982), for a further period of 5 days the levels of virus detected were similar to those in insects allowed only the 7 days MSV acquisition period.

In order to determine whether other leafhoppers can acquire MSV, various species were given acquisition feeds of 4-12 days. The leafhoppers were assayed in parallel with MSV-infected C.mbila. None of the leafhopper species tested contained concentrations approaching those in C.mbila, although some hybridisation could be seen with all species. This probably represents the quantity in the gut, as insects were tested immediately after feeding. (Table 1). When C.chinai was transferred to Zea perennis for 7 days following a 7 day access feed, no hybridisation could be detected.

Table 1 Hybridisation to a MSV probe in a number of leafhopper species

Species	Sex	Vector	a.a.p. (days)	Hybridisation	
				No of insects positive	*Degree
Cicadulina mbila	ND+	+	7	5/5	++++
" "	ND	+	0	0	0
" triangula	F	+	12	2/5	(+)
" chinai	F	-	7	3/5	(+)
Dalbulus maidis	F	-	7	2/5	(+)
Circulifer tenellus	F	-	4	2/5	(+)
" "	M	-	7	3/5	(+)
Macrosteles sexnotatus	F	-	7	4/5	+

*0 = no hybridisation, hybridisation = arbitary scale from (+) to ++++
+ND = not determined
a.a.p. = acquisition access period

Detection of Spiroplasmas

E.variegatus and C.tenellus were assayed for the presence of S.citri DNA 6 days after intrahaemocoelic injection of a culture of S.citri containing about $1x10^9$ spiroplasmas/ml. Non-injected leafhoppers, were assayed in parallel. All injected E.variegatus showed clear hybridisation. The haemolymph of individual leafhoppers given the same treatments as those assayed, was examined using darkfield microscopy. Spiroplasmas were observed in all the E.variegatus examined, but not in samples from C.tenellus, however hybridisation showed that 5 out of 9 were infected with spiroplasmas.

CSS DNA may also be detected in both D.maidis and E.variegatus. All E.variegatus leafhoppers showed positive hybridisation 5 days after intrahaemocoelic injection of a culture containing $2x10^8$ spiroplasmas/ml. D.maidis, tested after an acquisition access period of 5 days on corn stunt infected maize gave positive hybridisation.

DISCUSSION

This work was initiated to support a study of the epidemiology of MSV and to provide a rapid and sensitive assay system to enable the study of MSV transmission by its insect vector, C.mbila.

Using the squash blot (or "swat blot"), MSV DNA concentrations of 2.5pg may be detected, thus, assuming a DNA content of 20% by weight for MSV it is possible to detect 12.5pg of virus present in the vector. The method is rapid, sensitive, and requires only small amounts of the nucleic acid of the pathogen. 1ug of pure MSV DNA is sufficient for the production of a nick translated ^{32}P-labelled probe to assay at least 1000 leafhoppers. For quantification of virus concentration, areas of the nitrocellulose filter containing single leafhoppers may be excised and scintillation counted, spots of known concentrations of nucleic acid being used for comparison. In contrast, immunological methods require large

amounts of purified virus coat protein or membrane proteins for the production of antiserum and a significant time for its production, always provided that animal house facilities are available. ISEM requires electron microscopes and is time consuming and tedious for multiple samples. ELISA may require adsorption of the antiserum to prevent non-specific reactions. The swat blot technique, however, allows processing of a large number of samples with highly specific results. The nucleic acid used for probe production may be stored refrigerated for several years.

Provided radiolabelled probes of high specific activity can be prepared this technique should be applicable to all DNA-containing viruses, and with minor modifications, RNA-containing viruses, present in either leafhoppers, aphids or nematodes. The method has been shown to be more widely applicable, detecting the chromosomal DNA of corn stunt spiroplasma and S.citri in their insect vectors and the spiroplasma strain BC3 in the experimental leafhopper system, E.variegatus. These results suggest that the technique may be applicable to fungal or bacterial pathogens where chromosomal DNA may be used as a probe, and should therefore provide valuable information on the etiology of many pathogenic diseases associated with economically important crops.

In order to make spot hybridisation applicable to all laboratories it may be necessary to replace the radioactive probes by probes based upon chemically modified DNA, which can be detected using immunochemical methods (Langer et al 1981, Tchen et al 1984).

The main limitation to this method as a general screening technique is that probes to individual viruses may be too highly specific. Liquid hybridisation has shown that even closely related RNA viruses (as assayed by immunological and other criteria) have only limited amounts of base sequence homology (Zaitlin et al 1977, Gould and Hatta, 1981). However preliminary evidence from sequence analysis suggests certain regions maintain close homology. It should therefore be possible to isolate clones detecting a large number of strains and others which are strain specific. In fact, this specificity may provide a further advantage, allowing the detection of individual genes, or sequences when using cloned DNA probes. This may be especially useful for the investigation of those pathogens which multiply in their insect vectors. We are at present using strand-specific probes to investigate whether MSV replicates in C.mbila, and if so, in what organs. MSV is easily detected by squash blotting in dissected salivary glands.

Application of the technique to the acquisition and transmission of MSV, suggests that the method could be used to screen large numbers of insects collected from the field, to search for putative vectors based upon the quantity of pathogen they contain. Under laboratory conditions, possible vectors may also be identified by measuring acquisition of the pathogen from infected plants. In either case, the practicality of measuring pathogen concentration in large numbers of insects provides a new and powerful tool for those concerned with crop protection.

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VIROID DETECTION IN POTATO QUARANTINE: THE cDNA PROBE AND OTHER METHODS

P. S. HARRIS and C. M. JAMES

Department of Agriculture and Fisheries for Scotland, East Craigs, Edinburgh EH12 8NJ

A. D. LIDDELL

Department of Botany, University of Edinburgh, Edinburgh EH9 3JH

E. OKELEY (nee GOLENIEWSKA)

Present address: Department of Molecular Biology, University of Edinburgh, Edinburgh EH9 3JH

ABSTRACT

Testing for non-indigenous Potato Spindle Tuber Viroid (PSTV) affords protection for plant breeding and seminal material distributed to the British seed potato industry.
With possible exceptions, serological detection of the naked viroid RNA is not appropriate. Direct detection by polyacrylamide gel electrophoresis (PAGE) of nucleic acids is not sensitive. Most U.K. testing has used a sensitive bioassay of bulked samples where amplified infection in tomato test plants is diagnosed by PAGE. Currently a radioactively labelled cDNA probe (a gift from R. A. Owens, U.S. Department of Agriculture) is used in parallel with the older method. Modifications to sample preparation (de-naturing and/or target concentration) suggest a 5 to 100 fold increase in sensitivity, thus making the probe sufficiently comparable with tomato/PAGE. The probe can be used to test bulked samples or to unequivocally confirm infection in individual potato sources and has the advantage that tests can be completed within a week.

INTRODUCTION

Member States of the EEC are required to ban the introduction to their territory of certain harmful organisms including potato spindle tuber viroid (PSTV). There is a discerned threat, particularly to potato breeding programmes, from infection in several germ-plasm pools world-wide. Interceptions of PSTV infection have been made by the Department of Agriculture and Fisheries for Scotland (DAFS) over a fourteen year period. Current testing by DAFS and the Ministry of Agriculture, Fisheries and Food affords protection for plant breeding and seminal material distributed to the seed potato industry in Great Britain.

Serological methods are not appropriate for detecting naked viroid RNA with possible exceptions (Salazar, 1981). ELISA has been used to detect dsRNA and PSTV (Zanzinger and Tavantzis 1982).

The direct visualisation of potato and viroid nucleic acids on polyacrylamide gels following electrophoresis (PAGE) will not detect low concentrations of viroid and has not been used for most tests in the U.K.

DAFS current most sensitive test is by inoculation of potato nucleic acids to tomato and subsequently, after "amplification", by detection on polyacrylamide gels (PAGE of tomato leaf nucleic acids), (Harris and Miller-Jones 1981, with modifications, see Fig. 1). 24 x 50 tests can be set-up in a week (1200 individual plants tested in batches of 50) although the whole procedure takes 4 to 5 weeks. Cutting back tomato plants and allowing re-growth considerably enhances symptoms of mild isolates if that is desired although the PAGE result, not symptom expression is our diagnostic criterion.

Work is in progress on the use of a radioactively labelled cDNA probe. (The DNA sequence complementary to the entire 359 nucleotide RNA PSTV sequence has been inserted into M13mp9 phage (R. A. Owens, United States Department of Agriculture) which provides, when multiplied in infected bacteria, cDNA for a probe, Fig. 1). With the help of the Department of Botany, University of Edinburgh, the cDNA test with modifications has been used in parallel with existing tests since January 1984 in order to gain practical experience and give earlier clearance where possible.

MATERIALS AND METHODS

Phytosanitary arrangements and sources of viroid together with PAGE and inoculation of nucleic acid to tomato are essentially as described (Harris and Miller-Jones, 1981) with modifications to nucleic acid extraction, see Fig. 1 and below. One PSTV source originates in the USA and is provided from Poland, see below

Nucleic acid extraction using polyethylene glycol (PEG) follows Skrzeczkowski and Okeley, Potato Research Institute, Mlochow, Poland, see authors above, (manuscript in preparation).

Standard procedures are used for second strand synthesis from M13 phage DNA (see Fig. 1).

Use of a cDNA probe to hybridise with viroid RNA bound to a solid nitrocellulose filter is as described (Owens and Diener, 1981) with the substitution of a different salt and reducing buffer for sap clarification, (Palukaitis and Zaitlin, personal communication) and the addition of de-natured herringsperm DNA to hybridising media.

De-naturing of clarified sap and nucleic acid extracts follows Skreczkowski (personal communication) with reference to White and Bancroft (1982).

An outline of methods is provided in Fig. 1.

RESULTS

PAGE

Typically PAGE will not detect low comentrations of viroid although the two stage PEG method (Fig. 1) raises the viroid to host nucleic acid ratio.

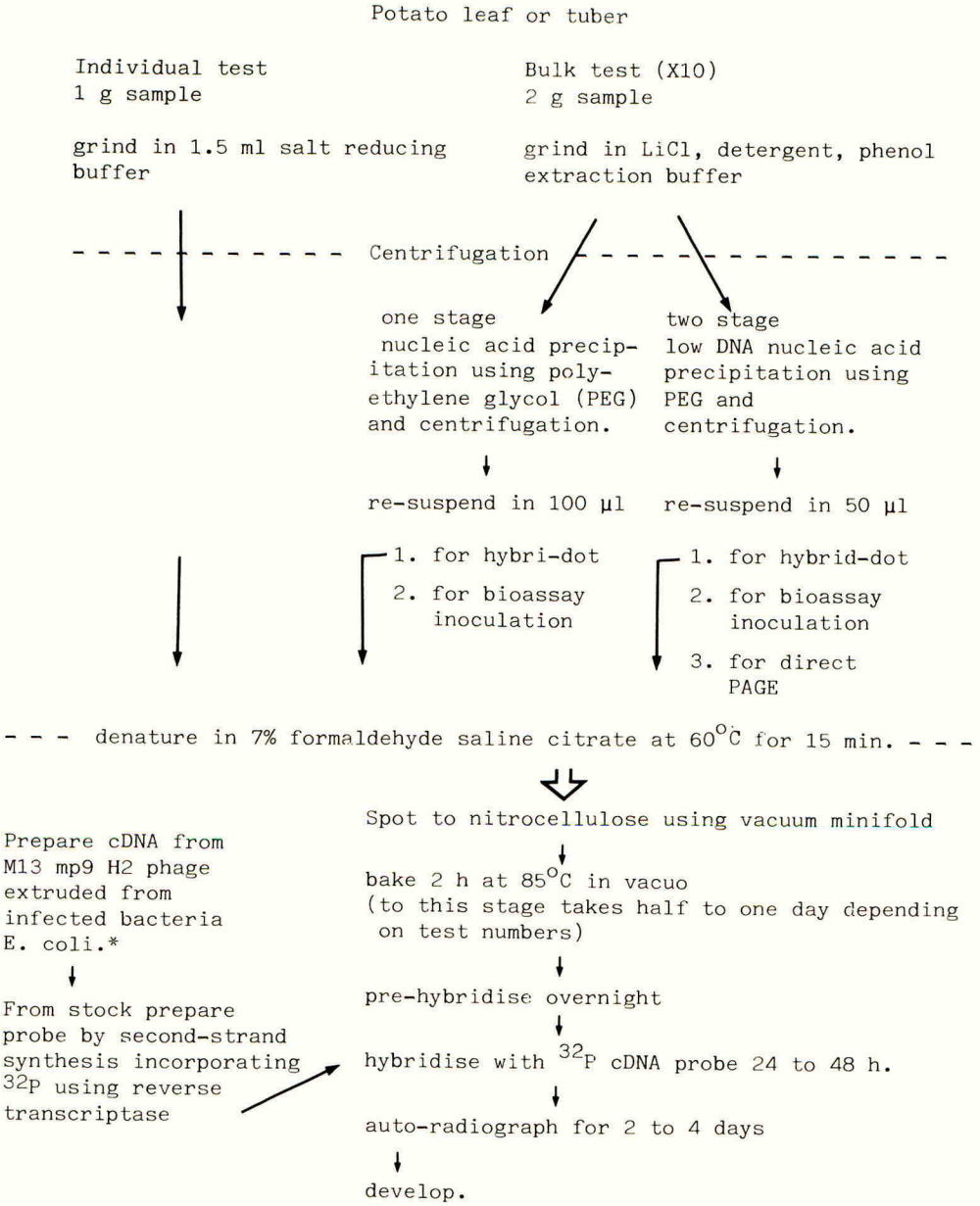

Fig. 1. Potato viroid test. Sample preparation for cDNA probing, bioassay or PAGE. Tubers yield less nucleic acid; increase sample X2 or X4.

*A total nucleic acid extract from transformed bacteria was tested for plant pathogenicity and found to be negative.

cDNA

Viroid in clarified sap from well infected potato or tomato leaf tissue is detected at a dilution end-point typically between 10^{-2} and 10^{-3}. The same sap de-natured and usually in the presence of 1 to 2.5 M NaCl will give a result at more than 10^{-3} on a dilution series.

Nucleic acid can be concentrated to give a greater density of target than can be obtained in a spot of sap. Typically, dilution end-point for detection is beyond 10^{-3} and, with de-naturing, beyond 10^{-4}. The detailed effects of salt concentration and the presence of an excess of host nucleic acid are being investigated (Skrzeczkowski, personal communication). Our results suggest successful detection of viroid at low concentration in the presence of an excess of host nucleic acid.

Non-specific reactions have not been detected for both clarified sap and nucleic acid extracts prepared as in Fig. 1. Extracts from healthy tomato leaves and several hundred potato leaves from many varieties have been tested. A more limited number of extracts from tubers were also negative. However, PEG precipitation of nucleic acids that had been extracted with phenol in a lower salt buffer (Harris & Miller-jones, 1981) gave faint non-specific reactions in some instances.

Bioassay

In direct comparison experiments dilution series of nucleic acids from infected plants are fully infectious to tomato seedlings at 10^{-4} or beyond.

DISCUSSION

Comparison of methods

A useful comparison of viroid detection by direct PAGE and by cDNA probes (probing target in solution as well as bound to nitrocellulose) is reported (Mohamed and Imperial, 1984). It is widely reported that viroid synthesis is much slower at temperatures below 20°C. Certainly viroid can exist at low concentrations (unpublished results). We have always required a greater (perhaps 100 fold) sensitivity in detection compared with PAGE.

Modifications in sample preparation (target concentration and de-naturing) suggest a promising increase in sensitivity for the cDNA probe. A rapid nucleic acid extraction technique (Fig. 1 Skrzeczkowski personal communication) has shown some promise in concentrating viroid in samples.

Improvements in the probe itself, possibly by using short synthetic probes, may be proven in the future.

At this date, the probe is sufficiently comparable with tomato/PAGE to be used to test bulked samples or to unequivocally confirm infection in individual potato sources and has the advantage that tests can be completed within a week.

Our work is mostly concerned with the hope of achieving repeatable results for large numbers of tests, and in particular being able to confirm that an effective procedure has been carried out in all instances.

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We wish to thank Dr R. A. Owens for the gift of M13mp9H2 and laboratory protocols. Drs P. Palukaitis, M. Zaitlin and R. H. Symons have provided protocols through Dr C. J. Leaver, Botany Department, Edinburgh University. Dr Leaver and his Department and Dr L. J. Skrzeczkowski, Mlochow, Warsaw, have provided support and essential information.

SECONDARY EMBRYOGENESIS AND THE PRODUCTION OF NOVEL DISEASE RESISTANT BRASSICAS

D.M. NEWSHOLME, M.V. MACDONALD, *C.-S. LOH, D.S. INGRAM

The Botany School, University of Cambridge, Downing Street, Cambridge CB2 3EA

*Present address: The Botany Department, The National University of Singapore, Kent Ridge, Singapore

INTRODUCTION AND OBJECTIVES

The phenomenon of secondary embryogenesis in winter oilseed rape (*Brassica napus* ssp. *oleifera*) and its use in the development of new disease resistant lines is discussed by Ingram *et al* (1984), and MacDonald and Ingram (1984, & this volume). The practical procedures employed are as follows: (a) the production of amphihaploid embryoids by anther culture; (b) the selection of secondary embryogenic lines; (c) the restoration of amphidiploidy by treatment with colchicine; (d) the regeneration of plants by adjustment of the levels of growth substances in the culture medium, and (e) the selection of somatic variants, especially those resistant to pathotoxins. These procedures are outlined below.

METHODS

Production of amphihaploids and selection of secondary embryogenic lines (Loh & Ingram 1982; Ingram *et al* 1984)

The first step in the generation of secondary embryogenic culture lines of *B. napus* is the production of anther embryoids. These develop from immature microspores and consequently have the haploid (= amphihaploid) chromosome complement, *n*=19. Buds of 3.0-3.5 mm are selected from winter rape cultivars and surface sterilised. The anthers are plated aseptically on to a defined medium of Keller & Armstrong (1978), with 10% sucrose, and subjected to a heat shock of 30°C. for 14 days in the dark. They are then incubated at 25°C. in darkness until the embryoids emerge. Young embryoids are removed and plated on the above medium with a reduced level of sucrose (2%) in continuous light for about 1 week. Finally, they are transferred to MS medium (Murashige & Skoog 1962), and maintained at 25°C. with a 12 hr. photoperiod.

When anther embryoids are maintained on MS medium for a period of 4-6 weeks, 40-80% develop abnormally and give rise to somatic secondary embryoids on their surfaces. The rest develop into haploid plantlets. The secondary embryoids have been shown

to develop from single epidermal cells of tissues derived from anther culture, and they develop through stages similar to those of sexual embryos (Plate 1). They are loosely attached to the parental tissue and can be transferred readily to fresh culture medium. A variable proportion of these in turn give rise to further somatic secondary embryoids. This pattern is repeated at each subsequent subculture.

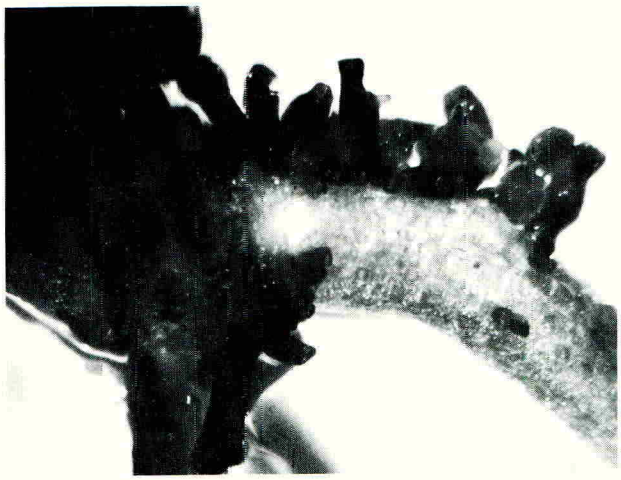


Plate 1. A secondary embryogenic culture of Brassica napus.

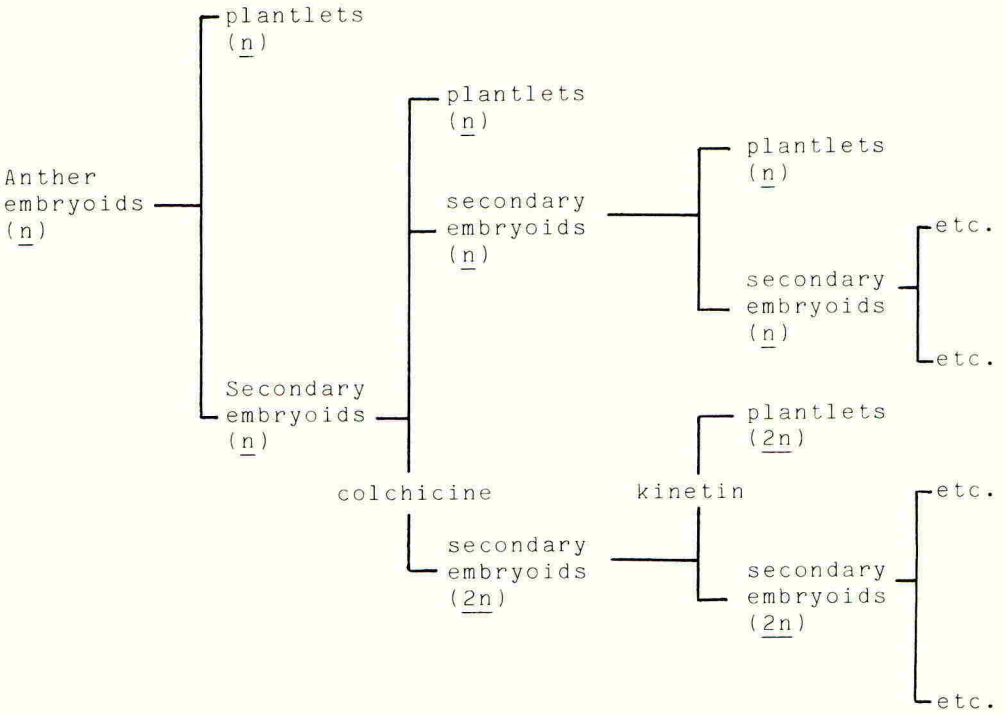
As the frequency of embryoid production through anther culture of oilseed rape is relatively low, and the flowering period is short, secondary embryogenesis offers a system to produce a large number of haploids throughout the year by repeated subculture, and the material is clonal.

Restoration of diploidy (Loh & Ingram 1983; Ingram et al 1984)

To study the genetics of plants derived from secondary embryoids it is necessary to restore the diploid state. To investigate this, embryogenic tissues and secondary embryoids were exposed to 0.02-0.2% colchicine in liquid MS medium for a period of up to 72 hrs., and then transferred to solid MS medium without additives. Chromosome counts of the root tips revealed a high proportion of diploids, although tetraploids and mixaploids were also observed. Best results were obtained with 0.05% colchicine for 72 hrs, and this treatment is now used routinely. The surviving tissues retain their embryogenic potential and give rise to further embryoids with stable ploidy levels.

Regeneration of plants (Loh et al 1983; Ingram et al 1984)

As the number of embryoids which develop into plants is unpredictable, a procedure for the regeneration of plants has been developed, by adjusting the levels of plant growth substances in the culture medium. Embryoids of 1-3mm were grown on medium supplemented with 10^{-7} to 10^{-4}M kinetin (6-furfurylaminopurine) alone and in combination with 10^{-7} to 10^{-4}M IAA (1-indoleacetic acid). Optimum results were obtained when secondary embryoids were plated on 10^{-4}M kinetin for up to 4 weeks, to trigger shoot development, and then transferred to medium without growth substances or with the addition of low levels of IAA to stimulate rooting. Plantlets produced in this way can be planted in compost. The ploidy of these plantlets is generally unaltered during the regeneration procedures. This means that embryoids of chosen ploidy may now be regenerated when required, as follows, to analyse the sexual heritability of variant characters.



Selection of variants (Loh 1982; Ingram et al 1984; MacDonald & Ingram, this volume)

Preliminary investigations of plants regenerated from anther embryoids and secondary embryoids has revealed variation in the following characters: a) the number of stems per plant, b) flower morphology c) time to flowering and d) levels of

resistance to disease. Several of these characters may have important implications in plant breeding, especially resistance to disease, if they are shown to be of a genetic nature. Detailed research on the generation of variants resistant to pathotoxins is being persued as follows.

(i) Leptosphaeria maculans

Secondary embryoids have been selected for resistance to Sirodesmin PL, Leptosphaeria maculans toxin, in culture media. These have been maintained in toxin free medium for one year and have recently been re-tested, as cultures against the toxin, and as regenerated plants against the fungus. Preliminary results suggest that plants regenerated immediately after selection, and those regenerated one year after selection were no more resistant than unselected material. This is in contrast to the results of Sacristan (1982), although mutagens may have been used in her work. The precise role of Sirodesmin in the host pathogen interaction is still unclear.

Currently, our work is centered on the use of genetically defined host-pathogen systems to identify the necessary cultural conditions required for an accurate assessment of all the components of resistance to L. maculans in B.napus tissue cultures. Differential resistance is being introduced into fast flowering lines (supplied by P.H. Williams, University of Wisconsin, Madison, U.S.A.) and callus cultures are being established to determine optimum conditions for the expression of resistance and susceptibility to L. maculans in parental material, callus cultures and regenerants.

ii) Alternaria species. (MacDonald & Ingram, this volume)

Preliminary studies with Alternaria species have shown that Alternaria brassicae and A. brassicicola produce non host-specific pathotoxins when grown in liquid culture. When added to MS medium, the partially purified toxins kill secondary embryoids (Plate 2). Research is currently being done to isolate and identify the pathotoxins, to determine their roles in infection and to develop in vitro techniques for detecting variants carrying resistance to the pathotoxins and to disease.

CONCLUSIONS

Secondary embryogenesis provides large numbers of clonal embryoids of oilseed rape throughout the year. Secondary embryogenic cultures have considerable potential for the development of clonal propagation systems, for the generation of somatic variants, and for the creation of new genotypes by genome transformation and mutagenesis.

It may be possible to develop in vitro disease screening procedures using pathotoxins, or by challenging tissues directly with fungi, once the cultural conditions which control the

expression of true genetic resistance are established.

The brassicas offer an excellent system for the study of genetics and biochemistry of tissue culture systems, and host-pathogen interactions, especially if fast flowering lines (Williams 1982), which flower within 19-29 days of sowing, are used.

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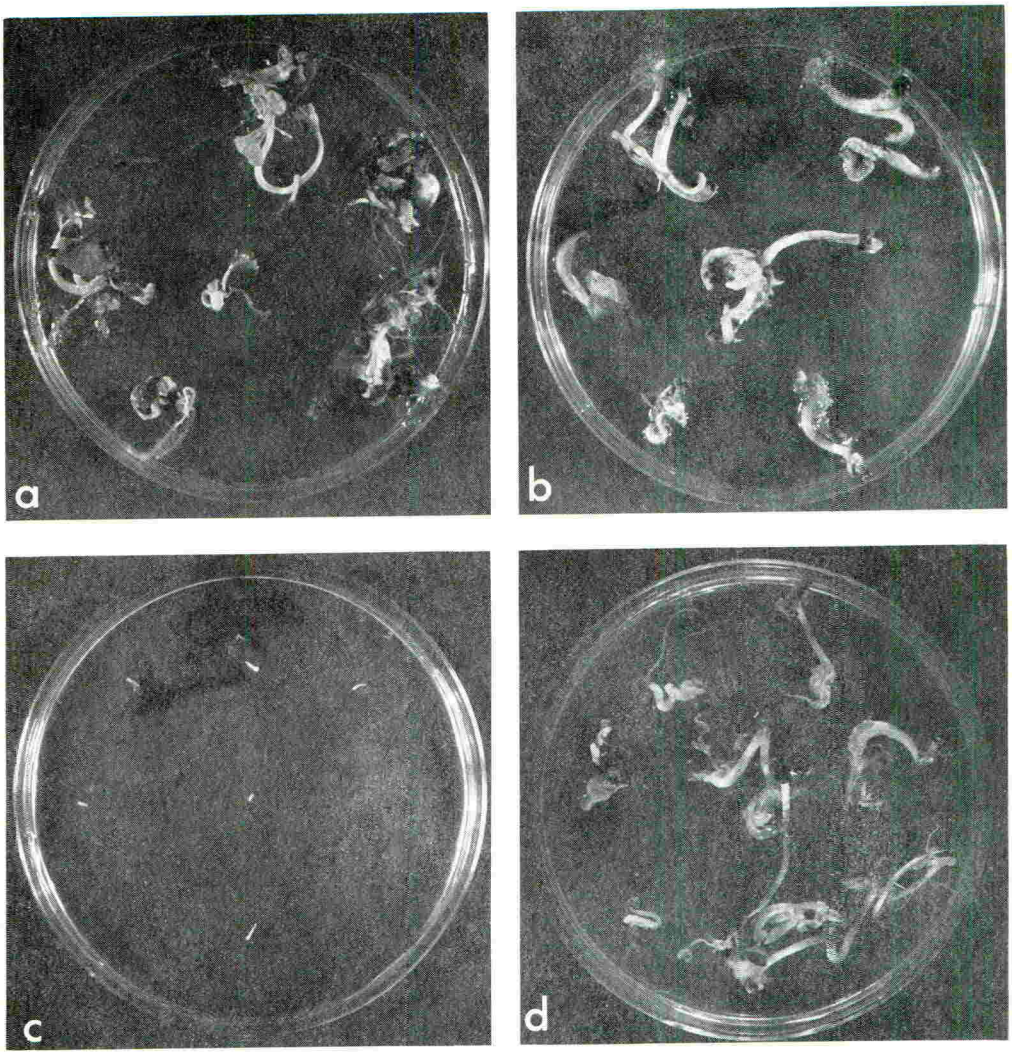

Plate 2. Secondary embryoids grown on MS medium for 4 weeks, with the addition of a) 10% distilled water, b) 10% extract of potato dextrose broth, c) 10% extract of Alternaria brassicicola toxin, d) autoclaved treatment (c).

IN VITRO SCREENING FOR DISEASE RESISTANCE

G. WENZEL, F. KÖHLER, R. SCHUCHMANN, W. FRIEDT, B. FOROUGHI-WEHR

Federal Biological Research Centre for Agriculture and Forestry, Institute of Resistance Genetics, Grünbach, Germany F.R.

ABSTRACT

Strategies for the incorporation of resistances into crop plants are demanding more and more often a quantitative approach. With this approach, procedures for rapid and reproducible screening are necessary. The paper describes to what extent in vitro cell procedures might permit selection of resistant types from within existing variation. Also, examples are given for the production of new variability and for the incorporation of haploids in resistance breeding programs. In potato, haploid calli and protoplasts are used in screening for resistance to viruses and fungi. In barley the regeneration from isolated microspores became possible, after conditioning the medium by ovaries. The regeneration rate is high enough, to use this system for selection. Fusaric acid was applied as a selective agent, and calli could be selected which survived 0.2mM fusaric acid, a concentration toxic to unselected material. In this procedure the advantages of a single cell system are combined with those of haploidy.

INTRODUCTION

The central steps in plant breeding are a) production of variability and b) selection. Today examples exist to show that in vitro techniques may increase the efficiency in both areas (Table 1). The two steps may also be combined, either partially or completely in vitro. One advantage of such in vitro procedures is their independence from climatic changes of the natural environment, making it easier to measure slight quantitative differences e.g. in response to diseases. With the increasing importance of horizontal resistances such techniques are becoming more and more important. Another one is the possibility to handle large numbers of individuals in a very small space and finally, work with microspores and haploids allows screening of single genomes, uncovering recessive traits.

SELECTION IN VITRO

A first step towards in vitro studies is the use of detached leaves or leaf pieces laying on agar in petri dishes, as e.g. in the screening for mildew resistance of rye (Lind 1983). An extension of such organ culture is the meristem culture, as in potato, which leads to rapid propagation of healthy material. If rapid propagation follows a pathway via stem embryogenesis, as e.g. in rape seed, this system of selection and propagation may be used even for the search of new variability (Hoffmann et al. 1982).

Production of new variability in homozygous starting material

We would first like to mention the present situation in our selection experiments for Phytophthora resistant potato clones starting from callus. These experiments were started by Behnke (1979) using unfractionated exotoxins of the fungus. Calli surviving on media containing the toxin in a concentration, which kills 90% of them, were transferred at least five times to identical toxic medium, before regeneration was induced. First tests for resistance were performed on greenhouse grown selected clones, and it could be demonstrated that the diameter of local lesions after mechanical inoculation with Phy-

TABLE 1

Breeding for disease resistant crop plants with in vitro techniques

Plants	Disease	Reference
a) without in vitro selection		
Sugarcane	Fiji-virus	Heinz et al. 1977
Potato	Alternaria	Shepard et al. 1980
	Phytophthora	Shepard et al. 1980
	Streptomyces	Jellis et al. 1984
	Y-virus	Jellis et al. 1984
	Leafroll-virus	Jellis et al. 1984
b) with in vitro selection		
Tobacco	Phytophthora	Helgeson et al. 1972
Potato	Phytophthora	Behnke 1979
	Fusarium	Behnke 1980
	Erwinia	Rehbein 1983
Maize	Helminthosporium	Gengenbach et al. 1977
Rape seed	Phoma	Sacristan 1982
Barley	Rhynchosporium	Branchard 1982

tophthora spores was significantly reduced compared with unselected control plants. The number of sporangia formed later did not differ, which means that only the infection rate was reduced but not the growth rate of the fungus or the sporulation speed. This resistance was unspecific, and is probably quantitatively inherited. From 42,200 calli 173 (0.4%) turned out to be resistant; from these 36 plants were regenerated which showed the resistance in the greenhouse test, and 34 of them were further screened in the field. Most of these clones became virus infected in 1981, and in 1982 Erwinia infected, as the starting material was only selected for its regeneration capacity, but not for virus or other resistances. Due to the weather conditions no Phytophthora appeared in 1983. In 1984 there was a heavy Alternaria attack, making selection for Phytophthora impossible (Table 2). So it is difficult to judge whether the results of this experimental series are encouraging. At present we use purified toxins which are extracted from the liquid fungal growth medium by ultrafiltration. The molecular weight of its active compound is in the range of 20,000 daltons (Stolle and Schöber 1984).

Similar experiments have been started to screen for Fusarium resistance in protoplast derived calli. Fusarium produces exotoxins, which inhibit the respiratory activity in potato cell suspension cultures. At a toxin-concentration, which is 100-500 times higher than the basic value for selection, the respiration drops to zero within 30 min. According to the rate of inhibition the activity of the toxin can be estimated in the selection medium. For screening of Fusarium resistance such standardized toxin solutions are used in a concentration which allows 1-5% of the protoplasts to form walls, to divide and to continue their growth. For Phytophthora the increase of shoot tip fresh weight in the presence of the toxin was used.

TABLE 2

Results of field tests of dihaploid potato clones after *in vitro* selection for *Phytophthora* resistance

Year	total	healthy	diseased	
			Phytophthora	others
1981	34	5	5	24
1982	31	7	-	24
1983	20	17	-	3
1984	26	5	-	21

In these selection systems, new characters are expected from a pregiven homogeneous genotype without any recombination event. This means one is hoping for somaclonal variation, or in other words, for mutations. It is currently under active discussion how far this *in vitro* induced variability can be successfully used in breeding programs. Shepard *et al.* (1980) found such a tremendous variability in potato clones from the tetraploid variety 'Russet Burbank' that they recommended the use of such protoclones for 'intracultivar improvement'. We regenerated more than 3,000 clones from dihaploid protoplasts and found some striking aberrants too (Wenzel 1980) but most of these turned out to be aneuploids without any practical value. The remainder were phenotypically uniform. Aneuploids are rather common and stable in the ordinary tetraploid potato. In a cross of e.g. 3x X 2x they can be found in large numbers and some are vigorous (Wenzel *et al.* 1984). It is generally accepted now that the amount of variation depends on the genotype and on the ploidy level.

One advantage of somaclonal variation, in comparison to simple mutation breeding, which did not achieve too much in applied terms in sexually propagated crops, is the chance to apply a selective pressure during the *in vitro* phase on a very large number of individuals. Prerequisite for this selection is a powerful screening system, e.g. an exotoxin of a pathogen; so the advantage of the pathogen in evolution based on its large reproduction numbers can be counterbalanced by handling large numbers of plant cells. It should be stressed, however, that there is no general need to increase the variability, as in most economic plants sufficient variability exists. Only for some specific traits this approach may become necessary. This means for tissue culture in general that there exists a need to prevent somaclonal variation rather than to increase it. At the moment the most recommendable procedure for stabilizing tissue cultures is a rather rapid passage through the *in vitro* phase. The environmental conditions have to be a compromise between too stressful *in vitro* conditions, which may cause additional alterations, and too rich a medium, which allows the survival of too many aberrants.

Selection in vitro within heterogeneous cell populations

The biggest sources of heterogenous cell populations are microspores from heterozygous parental plant. During their regeneration either within the anther or in pollen culture, only a small fraction of the total population will regenerate. At the moment there are hardly any techniques available to control the genotype of microspores and to regenerate only an interesting fraction. On the other hand natural selection is common (Hoffmann *et al.* 1982; Friedt *et al.* 1984). Besides this uncontrolled selection haploids offer advantages by

their simple genome accelerating selection. The present situation in using them will be described for the examples of potato and barley.

Solanum tuberosum

In potato the value of a stepwise reduction of the ploidy level with the advantage of a simpler inheritance to facilitate the combination of qualitatively inherited characters (Wenzel et al. 1979) is generally accepted today. With this approach we have worked with clones carrying the extreme resistance to potato virus X (PVX), which has been extracted from Solanum acaule and on the extreme resistance to potato virus Y (PVY), extracted from S. stoloniferum. Besides extreme resistance, field resistances are used for these two viruses and additionally for potato leaf roll virus (PLRV). In tests for virus resistance after reduction of the ploidy level, conversions of the resistance type were found. Either previously field resistant clones became extremely resistant or vice versa (Table. 3). Besides these resistant clones, completely susceptible clones segregated, as expected.

TABLE 3

Types of resistance in androgenetic 2x potato clones starting from extreme or field resistant donor clones

	P V X						P V Y					
	extr.res.			field res.			extr.res.			field res.		
number of 2x donor clones	7			11			5			11		
	e.r.	f.r.	susc.	e.r.	f.r.	susc.	e.r.	f.r.	susc.	e.r.	f.r.	susc.
number of androgenetic clones	1	1	5	1	1	9	2	1	3	1	2	8

e.r. = extreme resistance; f.r. = field resistance;
susc. = susceptible

Data are available now, which demonstrate that field resistance to PVY and PLRV is maintained and expressed throughout the process of ploidy level reduction and doubling over a five year period. This means that both, monogenically as well as additively inherited resistances and probably other agronomic traits are maintained during the successive haploidization processes. When a genome has passed through the monohaploid level, an additive character is homozygous and can be transferred efficiently via hybridization like a monogenic one. In a vegetatively propagated crop like potato, the new F_1 hybrid can be propagated as a new heterozygous stable clone. For PVX or PVY resistance this might not be too essential, but for PLRV, where no qualitative resistance is available, this might be very important in potato breeding.

Hordeum vulgare

In barley doubled haploid varieties are already on the market, though not coming from anther culture yet but from haploids derived from crosses to H. bulbosum (Ho and Jones 1980). We have followed predominantly the anther culture procedure, as the overall number of genotypes is larger in the microspore population, than in the smaller number of egg cells.

In our winter barley program we focus on a specific problem: the incorporation of resistance to the soil-borne barley yellow mosaic virus (BaYMV). The

winter barley crop in central and northern Europe especially, but also the spring barley crop in Japan are frequently damaged by this virus. Today, increasing barley growing areas are infected with BaYMV, as a consequence of which considerable yield losses occur. On the average, the grain yield of susceptible varieties can be reduced by 30 to 70% or even more, as compared to a resistant variety. Since the virus cannot be controlled reliably by chemicals, yield losses can only be prevented by growing resistant cultivars.

A breeding program with a haploid step offers a rapid approach for the incorporation of BaYMV resistance (Foroughi-Wehr and Friedt 1984). From microspores, more than 500 androgenetic lines were produced from F_1 anther donor hybrids form crosses of susceptible lines to the resistant cv. 'Franka'. Of 292 A-lines tested, 65% proved to be resistant to BaYMV. This shows that a character like BaYMV resistance can be efficiently and rapidly combined with other favourable characters via haploid techniques; it additionally demonstrates the time gain for this approach.

Quite often a linkage disequilibrium was observed. This influences the recommendation of which generation offers the most suitable starting material for haploid extraction. If linkage groups should be kept together, which means that only a few recombinations are desired, as e.g. in crosses between adapted varieties, one should start with F_1 material. If, however, frequent recombinations are necessary for breaking repulsion linkages, then the starting material should be an F_3, or if a good screening system is available possibly an F_2. But even for stable characters with rare recombinations it is not possible to predict, whether the final product will come up to the wishes of the breeder. In rape seed, e.g. good vitality of microspores was coupled with high glucosinolate contents in androgenetic regenerants. Most plantlets had a much higher glucosinolate content, than the starting material. The spontaneous selection worked against the desired breeding aim, low glucosinolate content. The reason for this selection may be that a high glucosinolate level favours spontaneous doubling. Similar situations may come up in screening for resistances in androgenetic offspring.

Such a selection amongst microspore populations would be either prevented or manipulated on a more reliable basis, if their culture in isolation from the anther could be obtained. Cereal microspores offer a handy system for isolated microspore culture, as they have a very thin exine, allowing the observation of early regeneration processes. In agreement with the data of Sunderland (1984), we have found that the culture medium can be conditioned by an undefined compound produced from the anthers and also from ovaries. In such conditioned media cell development continues and plants can be regenerated. From a total of about 50,000 uninucleate microspores suspended in such a medium, 0.2% divide and about 30% of these grow further to callus. In this system fusaric acid was applied as a selective agent at the microcallus stage in a concentration of 0.1 mM. Per 1,000 calli, 4 were found to be resistant, a rate rather similar to the selection success in the Phytophthora/potato callus system.

CONCLUSIONS

Here some speculation on further progress in using tissue culture as a tool in resistance breeding is necessary. In cell culture today, such a tremendous amount of empirism is encoutered that a strong need for basic research exists. It is, however, easy just to ask for more basic research and to expect an early constructive understanding of regulatory processes acting in a higher plant. In a diseased plant the situation becomes even more complicated, forcing us to follow for the immediate future a trial and error approach. Probably

the rapid progress in gene technology will help also this section of biotechnology leading to an improved understanding of basic processes, and their exploitation in a more predictable manner. Then cell culture will become an even more powerful step in plant breeding, especially where increasingly polygenically coded characters are used to produce a more durable resistance. Such resistances normally follow a quantitative inheritance, which demands quantitative tests. Here in vitro procedures with plants grown under artificial environment offer the highest reproducibility and consequently will be of increasing need for such new breeding strategies. The use of preselected microspores will facilitate the rapid incorporation and early identification of resistances based on additive gene effects. Thus, in vitro approaches will supplement classical techniques as well as real gene technology.

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RECOMBINANT DNA PROBES FOR DETECTION OF VIRUSES IN PLANTS

D.C. BAULCOMBE, R.E. BOULTON, R.B. FLAVELL, G.J. JELLIS

Plant Breeding Institute, Maris Lane, Trumpington, Cambridge CB2 2LQ, U.K.

ABSTRACT

A method has been developed, based on nucleic acid hybridisation, for the detection of plant viruses in crude sap samples. The probe for this test is produced by molecular cloning of viral sequence in bacterial plasmids. The sap samples are simply spotted onto nitrocellulose membrane, dried and incubated with the probe. The non-hybridised probe is then removed by washing. Currently, the probes are radiolabelled and hybridisation is detected by autoradiography although it is likely that non-radioactive labelling will be used in future. The sensitivity and accuracy of the method compares with ELISA, but the hybridisation method may be more specific for viral strains.

INTRODUCTION

The ability to detect plant viral infections is important in many areas of plant production. Depending on the virus, the procedure may involve an assay for characteristic symptoms or a biochemical method which specifically identifies the presence of virus. The latter usually involves an immunological reaction. This paper describes the development and application of an alternative biochemical method based on the hybridisation of radioactive DNA probes to plant extracts.

The method has been developed for the specific needs of potato breeders screening for resistance to potato virus X (PVX), potato virus Y (PVY) and potato leafroll virus (PLRV). Until recently, for PVY and PLRV, the methods involved infection of plants with virus. In the following year plants were grown out from tubers of infected plants to allow symptoms to develop. Potato clones which failed to show symptoms were retained as "resistant" lines and included in further selection steps of the breeding programme. For PVX, the assay involved side grafting an infected scion on to test plants. Immune plants do not show symptoms; field resistant plants may show a top necrotic hypersensitive response while susceptible plants may develop severe mosaic (Howard et al. 1977). Clearly these are labour intensitive methods, which require field space for two years, or for PVX, extensive glasshouse facilities. It should also be noted that symptom production may not be a reliable indication of infection, so that each clone needs to be tested in several replicates. Because of these considerations, screening for virus resistance is delayed until the fourth year of the breeding programme, by which time the 25,000 progeny of a cross have been reduced, by other selections, to a few hundred.

It was evident therefore that a replacement for these laborious methods would need to be rapid and straightforward so that large numbers of samples could be handled. In addition it would be useful if the methods could be applied to young tuber shoots or to primary infections so that the second year of growth to allow symptom production could be eliminated.

In many cases, assay methods based on the reaction of antibodies to virus-specific coat protein have been a successful answer to these problems. In particular, the enzyme-linked immunosorbent assay (ELISA) (Clark & Adams 1977), which employs an enzyme antibody-conjugate to detect the viral

antigen, has been widely used. However, ELISA may not be reliable for virus detection, particularly of PLRV, when used in large scale tests with tuber or sprout sap extracts from a range of different genotypes. Unidentified contaminants of sap samples may interfere with ELISA so that false positive or negative readings are produced.

It was anticipated that nucleic acid hybridisation methods would not be subject to the same interference, and in addition that methods would be used which are highly suitable for the manipulation of large numbers of samples.

AN OUTLINE OF HYBRIDISATION AND CLONING METHODS

A standard method of analysis for similarity between two or more nucleic acid samples involves nucleic acid hybridisation. One sample is immobilised on a solid support, often a nitrocellulose membrane and the second is radioactively labelled. Provided that both samples are rendered single stranded the radioactive probe will hybridise with homologous sequences on the support, under the appropriate conditions. Non-hybridised probes can be washed away and the extent of similarity determined by measurement of radioactivity retained on the filter.

In order to apply this basic method to the assay of plant viral RNA, it was necessary to prepare highly specific probes for hybridisation and to devise a method by which viral RNA in crude extracts would be immobilised in a form which allows hybridisation to occur.

In principle it would be possible to use the purified viral RNA directly as a template for production of a radioactive DNA probe, using the enzyme reverse transcriptase. In practice however this is not always suitable as it may be difficult to purify the viral RNA to be absolutely free of cellular RNA species, and the resulting probe would not be completely viral specific. This is a particular problem with viruses, including PLRV, which occur at low levels in infected tissue. Fortunately, the techniques of molecular cloning allow the production of completely specific probes from only partially pure viral RNA. Figure 1 describes the steps of this procedure. In step 1, the single stranded RNA is copied into the opposite strand of DNA with reverse transcriptase. This is then converted into double stranded DNA by DNA polymerase (step 2) and tails of oligo dC added with a third enzyme, terminal deoxynucleotidyl transferase (step 3). In steps 4 and 5 a bacterial plasmid molecule is prepared for use as a vector for cloning of the double stranded DNA. The plasmid is cleaved at a defined point using a restriction endonuclease (step 4) and tails of oligo dG added (step 5) again using terminal deoxynucleotidyl transferase. The dG tails of the plasmid are then annealed with the dC tails such that a circular molecule is generated which can be transformed into, and replicated in E. coli cells (step 6). Each plasmid will take only a single insert, and each E. coli cell will accept only a single plasmid molecule. Thus any one colony of E. coli cells contains only a single type of insert in the plasmid. The identity of inserts as copies of PVX-RNA, PVY-RNA or PLRV-RNA was confirmed by showing that the insert hybridised to an RNA molecule of the same size as the viral RNA and which was absent from non-infected tissue (Baulcombe et al. 1984).

The plasmid DNA may be prepared easily in milligram quantities using standard procedures and the insert excised and purified by restriction endonuclease action and agarose gel electrophoresis. A straightforward method of radiolabelling DNA, and which is applicable for the production of highly labelled probes for hybridisation is the procedure of nick translation

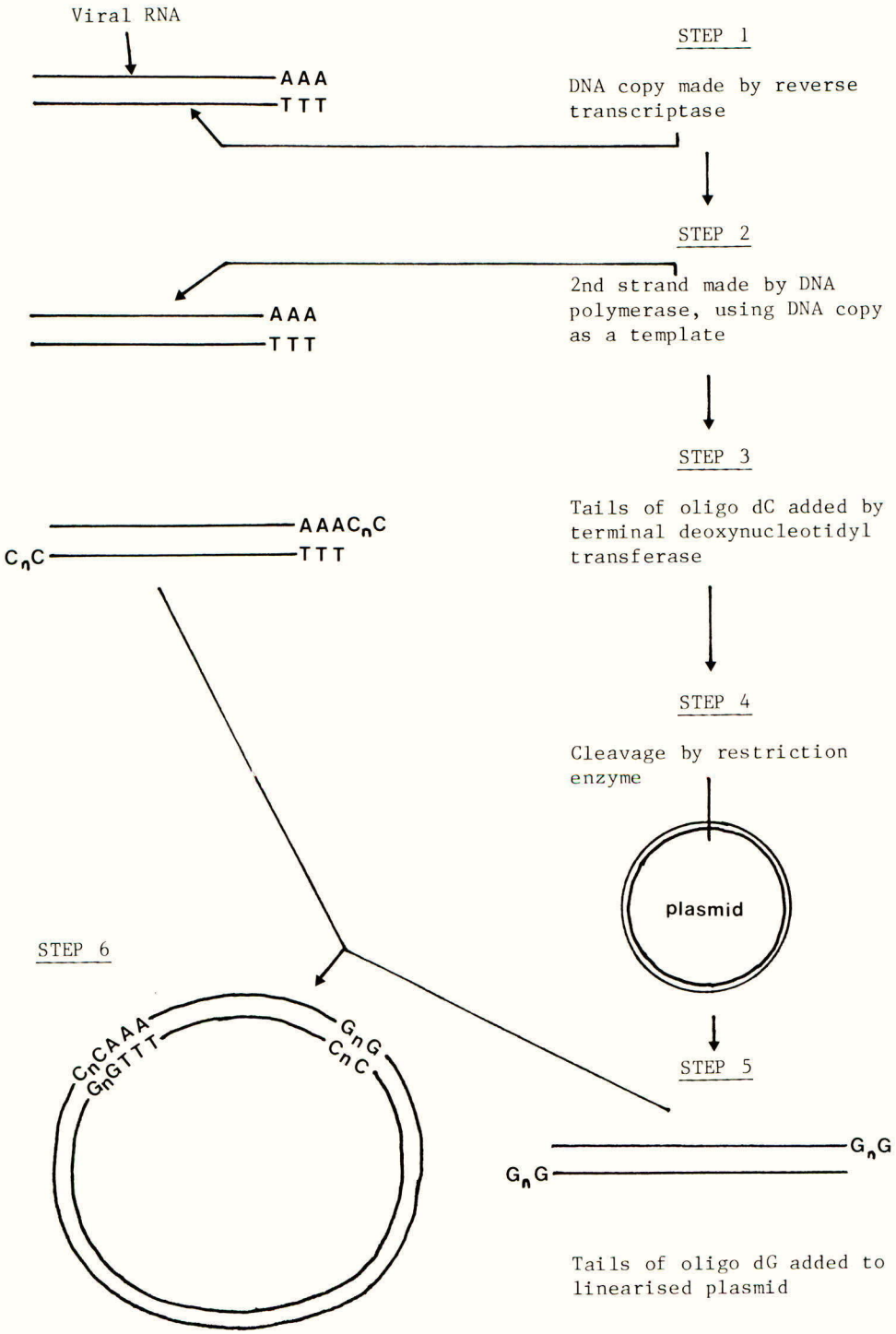

Figure 1. The steps in molecular cloning a DNA copy of an RNA sequence.

(Rigby et al. 1975). This uses the 5'-3' polymerase and the 5'-3' nuclease activities of E. coli DNA polymerase and the ability of DNAse I to introduce single stranded nicks into double stranded DNA. DNA incubated in the presence of these two enzymes and radioactive deoxynucleotide triphosphates is degraded on one strand from the site of nicking by the exonuclease activity, but then repaired by the polymerase action using the radioactive nucleotides. With ^{32}P labelled nucleotides it is possible to obtain specific activities of $>10^8$ cpm μg^{-1} DNA with this method. Of course, these double stranded DNA probes need to be denatured before starting hybridisaton.

Before this work was started, it was known that the DNA probes would hybridise to purified viral RNA which was immobilised on nitrocellulose. It was further known from the work of Owens and Diener (1981) that the basic methods would detect viroid RNA in crude sap samples which were immobilised on nitrocellulose. However it was not clear whether it would be possible to detect the RNA of encapsidated virion particles. A series of preliminary experiments were carried out to investigate this, in which crude samples of sap were dried onto nitrocellulose either untreated, or subjected to treatments designed to disrupt the virion particle. In fact, the results indicated, with samples of PVX-infected plants, that the strongest hybridisation signal was produced by untreated samples (Baulcombe et al. 1984). Further controls with purified virus showed that this was not because the samples contained non-encapsidated viral RNA, but because the various treatments to the nitrocellulose membrane, either before or during hybridisation, rendered the viral RNA available for reaction with the probe. The method which is used simply involves the application of 1 µl of crude sap from the test plants onto a nitrocellulose membrane which has been treated with a high salt solution. The membrane is then dried to fix the samples and subjected to the hybridisation. Each membrane can accommodate many samples at a density of 1 cm^{-2}. Following hybridisation, the non-hybridised probe is washed away and the presence of hybridised probe detected by autoradiography. Figure 2 illustrates the data obtained by this method and shows the analysis of eighty-four samples of sap hybridised with a probe for PVX and illustrates a number of positive and negative samples.

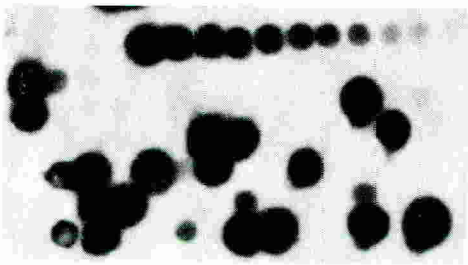

Figure 2. Hybridisation to crude sap samples. Eighty-four different and independent samples of sap from potato plants in a breeding programme were immobilised on nitrocellulose, hybridised with [^{32}P] probe for PVX and autoradiographed. Samples in the top row represent a doubling dilution series of infected plant sap in healthy plant sap.

THE APPLICATION OF HYBRIDISATION TO DETECT VIRUS IN CRUDE SAP SAMPLES

Subsequently, experiments were carried out to investigate the sensitivity, reliability and specificity of the assay method in comparison with ELISA and symptom assays. The sensitivity was investigated by dilution of a sample of purified virus in crude sap of non-infected plants or in water (Baulcombe et al. 1984). This showed that the hybridisation assay was equally as sensitive as ELISA and also that crude sap components had only a slight adverse effect on the degree of hybridisation.

The reliability of hybridisation as a routine method was assessed by comparison of data obtained with the results of ELISA and symptom assay. The data, shown in Table 1 for PVY and PLRV confirm almost complete agreement between the two biochemical tests. There was only loose agreement between symptom production and these tests. It is therefore concluded that the biochemical tests are similarly reliable as a means of virus detection and more reliable than symptom production.

TABLE 1

Detection of PVY and PLRV in leaves

Hybridisation	+	+	-	-	+	+	-	-
ELISA	+	+	-	-	-	-	+	+
Symptoms	+	-	+	-	+	-	+	-
PVY	11	8	17	36	0	4	0	0
PLRV	10	8	4	37	13	17	0	1

The specificity of virus assay by hybridisation was of particular interest in view of the high degree of sequence polymorphism which is known in isolates of plant RNA viruses. In addition it was expected that, since hybridisation probes were likely to include regions outside the viral coat-protein gene, that the reaction between strains would differ from that shown by immunological methods which consider only the coat-protein. As a model for this investigation, two strains of PVX were compared from PVX group 3 and PVX group 2. These were strains which showed strong immunological cross-reactivity. However, cross-hybridisation showed that there was only weak similarity at the nucleic acid level between these strains. Furthermore, it was shown by analysis of different fragments produced by restriction enzyme digestion of the cloned PVX sequences, that this weak cross hybridisation was localised on the viral genome. Certain regions of up to several hundred nucleotides were shown to be free of cross hybridisation. This example suggests that hybridisation is a more strain-specific technique than immunological methods. However this point clearly needs to be investigated in more detail for different viruses. For example, a comparison of PVY strains, PVY^{O} and PVY^{N} which are serologically very similar, has also failed, so far, to distinguish between these strains by hybridisation methods (unpublished).

CONCLUSIONS

A simple and standard nucleic acid hybridisation technique can be used, virtually unmodified, for the detection of encapsidated viral RNA in crude extracts of potato plants. In terms of sensitivity and reproducibility the

hybridisation is good as ELISA and definitely superior to the assay of symptom production. In addition the logistics of the hybridisation techniques permit the handling of large numbers (hundreds) of samples with ease. It is likely therefore that this method of virus detection will find application in breeding programmes, seed and plant production units and quarantine stations where many plants need to be tested.

At the time of writing the sequences from a number of RNA viruses including tobacco rattle virus, cucumber mosaic virus, tobacco mosaic virus, cowpea mosaic virus, alfalfa mosaic virus and brome mosaic virus have all been cloned (unpublished data, DCB and various pers. comm.) in addition to those described above. Application of the hybridisation assay to other viruses requires the production of a wider range of probes. However, it should be noted that in a suitably equipped laboratory, the production of DNA clones is very straightforward. Because of the nature of the cloning step, the production of pure probes does not depend on the prior purification of the virus. The availability of probes is not, therefore, a barrier to the application of hybridisation as a means of virus detection.

The use of radioisotopes, which does limit hybridisation to specially equipped laboratories, is likely to be discontinued as techniques are developed further for non-radioactive labelling of nucleic acids (Leary et al. 1983). Use of the non-radioactive label is limited at the moment, by a lack of sensitivity and by non-specific reaction in the components of crude sap (unpublished data). It is likely however that these problems can be overcome, perhaps assisted by new methods which allow the production of single stranded probes (Little 1984). Unlike the double stranded DNA probes described in this paper, the single stranded probes do not self-reassociate during the hybridisation and therefore remain single stranded and available to hybridise with the sample. Detection limits as low as 10^{-15} g have been reported (Little 1984), an improvement of 10^3 in sensitivity over previous methods.

The strain specificity of hybridisation probes will need to be checked for each particular virus. The comparison of PVX[2] and PVX[3] illustrates that in some cases it may be necessary to select as probes certain regions of the viral genome which show less strain variation. For other applications, where extreme strain specificity is required, it will be necessary to select more variable regions. This property of probes can be seen therefore to offer versatility which is not available from serological methods, but which needs to be carefully controlled.

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MOLECULAR GENETICAL ANALYSIS OF BACTERIAL PATHOGENICITY TO PLANTS

M. J. DANIELS

John Innes Institute, Colney Lane, Norwich NR4 7UH, Norfolk, UK

ABSTRACT

Plant pathology is beginning to benefit from recent technical developments in molecular genetics which can be applied to most Gram-negative bacteria, including plant pathogens. Recent successes in cloning genes whose products are involved in symptom production and host specificity in Pseudomonas and Xanthomonas spp. are described, bringing the hope that it may soon be possible to describe in detail the biochemical basis of these phenomena.

INTRODUCTION

The processes by which invading pathogenic microbes induce disease in plants are poorly understood, despite the considerble efforts expended in research in plant pathology over the past century. As Preece (1982) has pointed out, the basis of many of the most obvious macroscopic features of disease is unknown. The disease phenomenon must be a complex process, involving an intimate interaction between at least two distinct organisms (the host and the pathogen), and probably more if vectors, saprophytes and antagonistic microbes are taken into account. Because of these close relationships, variation which may from time to time have arisen in one member will have tended to result in the selection of corresponding variants in the others, so increasing the complexity at the genetic level.

Despite the difficulties it is necessary to try to dissect and understand pathogenicity in detail, not only because of its intrinsic scientific interest but also because longer term strategies in crop protection may depend on knowledge of the interacting biochemical components of the plants and pathogens. Present chemical controls depend on relatively crude properties such as inhibition of pathogen growth, and breeding of resistant plants probably exploits only a small fraction of the possible genetic variation. One may hope that it will soon be possible to identify components involved in key biochemical interactions required for successful parasitism, which will then be targets for site-directed mutagenesis using the procedures for genetic manipulation of plants which are at an advanced stage of development (see for example Kosuge et al. 1983).

One of the striking features of modern biology is the power of genetic analysis (essentially an abstract activity) in defining the properties of complex biochemical systems. A "classical" example is provided by the work of Jacob and Monod (1961) who deduced the nature of the control of inducible enzyme synthesis in bacteria by genetic methods, to see their predictions confirmed biochemically some years later. Faced with the complexity of plant disease it is natural to ask whether genetics can help us, and it is the purpose of this paper to highlight some recent advances which hold promise for the future.

In the past decade the power and scope of genetics has been greatly increased by the steady development of the set of molecular genetical procedures generally called "recombinant DNA technology". The core of this activity is the "cloning" of genes, which involves the fractionation of

genomes in a highly specific manner and the collection and propagation of the fractions in a versatile microbial host such as Escherichia coli. Having achieved this it is a straightforward matter to purify physically the genes of interest in large quantities. It is noteworthy that whereas biochemical purifications usually start with large quantities of material and end with minute amounts of the purified end-product, the reverse is true of gene purification. The intermediate steps of cloning can be carried out with microgram quantities of material and the pure end products can be amplified without limit. The cloning or isolation of genes is not of course an end in itself. It does however provide a substrate for further analysis. For example, it is possible to determine the complete nucleotide sequence of the genes, which in conjunction with in vivo or in vitro protein synthesis studies enables the primary structure of the gene products to be deduced. In turn this may allow the isolation on a preparative scale of the native products for further analysis. The nucleotide sequence will reveal the regions controlling the expression of the genes, and by further cloning experiments the quantitative expression can be varied within wide limits; moreover highly specific mutations can be introduced, and the effect of all these changes on the function of the genes and their products observed. The new technology has brought about almost a qualitative change in the type of experiment that can be undertaken and the amount of information which can be generated is awesome. General accounts of genetic manipulation are given in a number of text books and review articles, e.g. Old and Primrose (1981) and Timmis (1981), while detailed experimental protocols have been compiled by Maniatis et al. (1982).

EXPERIMENTAL APPROACHES

The choice of organism

Since molecular biological studies of plant-pathogen interactions are at an early stage of development where the fundamental properties of the systems still remain to be defined, priority may need to be given to the experimental suitability of certain host-pathogen combinations, rather than to their economic importance. As with all genetical experiments it may be necessary to work with large numbers of individual plants and pathogen clones. Consequently pathogenicity tests must be easy to perform and give unequivocal results in a short time, using plants of defined genotype which are easily and inexpensively grown.

Some plant viruses have been studied by molecular genetical techniques, particularly DNA viruses such as cauliflower mosaic virus (Hull & Covey 1983). However the obligately parasitic nature of viruses imposes some experimental restrictions. Fungi are the most important plant pathogens in Europe, but molecular genetics of plant pathogenic fungi is in its infancy (Yoder 1983). Some progress has been reported with a few species of saprophytic filamentous fungi (e.g. Neurospora, Hughes et al., 1983; Stahl & Lambowitz, 1983) and it is to be hoped that techniques such as those perfected for yeast (Struhl 1983) will become more generally applicable in the near future.

At present Gram-negative bacterial pathogens (i.e. Erwinia, Pseudomonas and Xanthomonas spp.) offer the best prospects of success and the rest of this paper will be devoted to discussing recent progress in this area.

Mutagenesis

Genes are usually identified initially by the effects of mutation on some property of the organism. In the case of pathogenicity or virulence genes two approaches have been used. Where there is reason to suppose that a particular pathogen product such as a pectolytic enzyme is a pathogenicity factor, it may

be possible to devise procedures for identifying bacteria with mutations in the corresponding genes, enabling the role of the suspected factor in disease to be studied (Chatterjee & Starr, 1980; Staskawicz et al. 1983a,b). Obviously this approach cannot be used if the nature of the factors is unknown, and the alternative approach (which has been rarely used until recently) must be adopted of screening mutagenised bacteria directly on plants and recovering any mutants which show altered disease patterns (Daniels et al., 1984a; Boucher, 1984; Panopoulos et al., 1984). A special class of "mutant" consists of races of pathogens which are distinguished according to their ability to incite disease in differential host cultivars. However, since different races are usually natural isolates, they will not be nearly-isogenic and caution must be exercised in comparing them.

Many techniques for chemical and physical mutagenesis are available (Hopwood, 1970) but mutants induced by insertion of transposons have many advantages (Kleckner et al., 1977). Transposons are pieces of DNA which can move from one site in a DNA molecule to another (either within the same molecule or to another physically separate molecule in the cell). If a transposon inserts within a gene it causes a complete loss of function of the gene. Since some transposons (e.g. Tn5, Berg & Berg, 1983) insert at random sites, a wide spectrum of mutations can be obtained. Many transposons have been characterised physically in considerable detail, and the mutant site can therefore be identified physically by using probes specific for the transposon sequence. Moreover, many prokaryotic transposons themselves carry genes which encode proteins conferring antibiotic resistance on the bacterium harbouring them (e.g. Tn1, Tn3-ampicillin, Tn5-kanamycin, Tn9-chloramphenicol, Tn10-tetracycline) so that the insertion mutation is absolutely linked to an easily-scorable character, namely antibiotic resistance. This is a considerable advantage in subsequent genetic analysis for it is obviously easier to score large numbers of progeny from a cross for antibiotic resistance than for a property such as pathogenicity.

The general procedure for performing transposon mutagenesis is to introduce into the target cell a transposon-containing replicon (plasmid or phage) which cannot establish itself stably under certain non-permissive conditions. If under these conditions selection is maintained for the antibiotic resistance encoded by the transposon, the only colonies which can grow are derived from cells in which the transposon has jumped from the delivery replicon into the target cell's chromosome (or indigenous plasmid). A number of systems have been devised and it is a matter of trial and error which will be best for a new application. Selection of the isolate of a target species may influence the outcome (Boucher et al., 1981), while some species may be refractory to transposon mutagenesis (Turner et al., 1984), and caution is needed before concluding that the procedures have worked as anticipated (Meade et al., 1982). If direct transposon mutagenesis of a bacterial species proves difficult, it may be possible to mutagenise cloned DNA in E. coli, subsequently reintroducing the mutated fragment into the pathogen (see below).

Cloning techniques

Procedures which have been used for gene cloning in plant pathogens are very similar to those used for other bacteria, details of which can be obtained from the references given above. However, the choice of cloning vector plasmid is of some importance. Vectors commonly used for E. coli such as pBR322 cannot be transferred into unrelated bacteria because the ColE1 replicon on which the vector is based has a narrow host range. Certain groups of plasmids are unusual in that they can be transferred to and maintained by a wide range of Gram-negative bacteria, including Erwinia, Pseudomonas and

Xanthomonas spp. A number of cloning vectors have been constructed from P, Q and W incompatibility group plasmids (Ditta et al., 1980; Friedman et al., 1982; Bagdasarian et al., 1981; Frey et al., 1983; Tait et al., 1983) and with these vectors DNA from plant pathogens can be cloned in E. coli, and therefore be accessible to the considerable range of manipulations possible with that organism, but moreover the cloned DNA can be transferred back into the pathogen for functional testing. One of the most widely used vectors is pLAFR1, originally used for Rhizobium (Friedman et al., 1982). This vector is a cosmid (Collins and Brüning, 1978), allowing it to accommodate large fragments of inserted DNA (up to 30kb); in consequence the number of clones which have to be screened to recover any given gene is reduced.

RECENT PROGRESS

Space does not permit a comprehensive review of the increasing activity in molecular plant pathology. Undoubtedly the plant pathogen which has received most attention from molecular biologists is Agrobacterium tumefaciens, but since aspects of the biology of this organism are discussed elsewhere in the Conference and since recent reviews are available (Van Montagu and Schell, 1982), I shall not consider it here.

Pseudomonas solanacearum

Globally Ps. solanacearum is the most destructive bacterial pathogen. For some time it has been believed that the extracellular polysaccharide (EPS) produced by the organism is involved in disease, but in the absence of genetic analysis this could not be proved. Staskawicz et al. (1983a, b) isolated Tn5 insertion mutants which failed to produce EPS. Tn5 (which has a length of 5700 base pairs) has no site for the restriction endonuclease EcoR1. Consequently if the DNA of Tn5 mutants is digested with EcoR1 and the fragments cloned in a suitable plasmid in E. coli (which is simply achieved by selecting for kanamycin resistance) the insert DNA in the clones will contain not only Tn5 but flanking regions from the Ps. solanacearum genes into which the transposon inserted. The clone can therefore be used as a molecular (hybridisation) probe for isolating the wild type gene from a genomic DNA library (a pooled collection of E. coli colonies each carrying a separate fragment of the Ps. solanacearum genome, which together include the whole genome). The cloned wild-type EPS biosynthesis genes were then mutagenised with Tn5 and the mutant plasmids transferred into wild-type Ps. solanacearum, and by the process of marker exchange (Ruvkun & Ausubel, 1981) the Tn5 insertions were transferred into the corresponding sites in the chromosome. The new mutants were EPS$^-$ and non-pathogenic, indicating conclusively that EPS is a requirement for disease production, because it could be demonstrated in each case that a single mutation was involved.

Boucher et al. (1984) isolated a large number of Tn5 mutants of Ps. solanacearum and tested them individually on tomato seedlings. A number were found to have lost pathogenicity, and a comparison with the proportion of mutants which were auxotrophic suggested that a considerable number of genes are involved in pathogenicity (perhaps 50-100).

Pseudomonas syringae

Ps. syringae pv. glycinea has a well characterised race structure which can be studied using differential tester cultivars of soybean. Using the vector pLAFR1 Staskawicz et al. (1984) prepared a genomic DNA library from race 6 and transferred the plasmid pool en masse into a strain of race 5. Individual transconjugant colonies (each harbouring a different recombinant plasmid) were then tested on the cultivar Harosoy, on which race 5 but not

race 6 is virulent. One out of 650 colonies had acquired avirulence towards cv. Harosoy. When the recombinant plasmid from this colony was transferred into a Ps. s glycinea strain which is virulent on all soybean differentials, the transconjugants now acquired the virulence/avirulence pattern of race 6, and moreover transfer of the plasmid to Xanthomonas campestris pv. glycinea which is normally virulent on all differentials conferred race 6 specificity. Subcloning and Tn5 mutagenesis of the cloned DNA have localised the avirulence gene(s) to a 2-3kb region (Staskawicz, 1984).

Panopoulos et al. (1984) and Willis and Panopoulos (1984) isolated Tn5 mutants of Ps. syringae pv. phaseolicola and pv. syringae which were avirulent on bean. The mutants fell into 3 classes, one of which was avirulent on both pods and leaves, one was avirulent on pods but gave normal leaf symptoms, and the third was not only avirulent on bean leaves but also failed to give a hypersensitive reaction on non-host plants such as tobacco. The wild type genes corresponding to the mutants are being cloned for further analysis. There was no obvious correlation between the behaviour of mutants on plants and their ability to produce phaseolotoxin.

Xanthomonas campestris

Daniels et al. (1984a) screened large numbers of colonies of mutagenised X. campestris pv. campestris on turnip seedlings and isolated a number of non-pathogenic mutants falling into several classes. These mutants arose at a frequency approximately one-tenth the frequency of auxotrophs in any given mutagenesis regime, suggesting that a considerable number of genes is involved in pathogenicity (cf. Ps. solanacearum, above). A genomic library of wild-type DNA was prepared in the vector pLAFR-1 and mated en masse into selected mutants; single colonies were then tested for restoration of pathogenicity. Two recombinant plasmids have thus been isolated which complement separate pathogenicity mutants. One appears to carry genes involved in extracellular enzyme production, but the function of the other is unknown. The latter plasmid has been mutagenised with Tn5 and the mutations recombined into the wild type chromosome (cf. Ruvkun and Ausubel, 1981), so creating a new set of mutants clustering around the original site. When these mutations were mapped physically and tested for pathogenicity it was found that there is a region of at least 10kb in which any mutations give a non-pathogenic phenotype, probably indicating a linked set of genes (Daniels et al., 1984a,b). Detailed studies of the structure and expression of the X.c. campestris pathogenicity genes are continuing.

Gabriel (1984) has studied the basis of race specificity of X. campestris pv. malvacearum for differential cotton cultivars. A genomic library of race H (which is avirulent on certain cultivars) was prepared using the plamid pSa747 (Tait et al., 1983) and introduced into race K which is virulent on all the differentials employed. Clones were identified which rendered the race K recipients avirulent on certain cultivars.

DISCUSSION AND CONCLUSIONS

Although molecular genetical studies of bacterial pathogenicity have only recently been undertaken the results are encouraging and promise a significant increase in our understanding of the disease process. Recent findings concern both the mechanisms by which disease symptoms are produced on plants and the basis of host-pathogen specificity. It is particularly noteworthy that both Ps. s. glycinea and X. c. malvacearum appear to have genes for specific avirulence, analagous to the situation which prevails with many fungal pathogens (Day, 1981; Ellingboe, 1981). It is probable that the dominant

avirulence allele specifies a gene product which interacts with a host component to provoke defence reactions limiting pathogen growth. The availability of cloned avirulence genes opens up the possibility for a direct attack on this hitherto elusive problem.

It was suggested earlier that knowledge of pathogenicity and virulence factors would help to probe the plant resistance mechanisms, thereby suggesting new pathways for research in disease control. Complementary studies are also beginning on a more direct approach to cloning plant resistance genes (see, for example, Kuhn et al., 1984). One may hope that breeding plants for disease resistance will be revolutionised in the forseeable future.

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SECONDARY EMBRYOGENESIS AND SELECTION FOR RESISTANCE TO DISEASE IN OILSEED RAPE

MARY V. MACDONALD, DAVID S. INGRAM

Cambridge University Botany School, Downing Street, Cambridge, CB2 3EA

ABSTRACT

Many plant tissue culture techniques are now being used in the improvement of crop plants, including oilseed rape. Some tissues derived from anther culture of *Brassica napus*, develop secondary embryoids on their surfaces. These secondary embryoids are described and *in vitro* procedures for the selection of lines resistant to *Leptosphaeria maculans* and *Alternaria* spp. are outlined. The possibility of extending these techniques to other pathogens is considered.

INTRODUCTION

Various plant tissue culture techniques are now being used in the improvement of crop plants. These include embryo culture, *in vitro* fertilisation, anther culture, *in vitro* mutagenesis, the regeneration of somatic variants, somatic hybridisation by protoplast fusion and genome transformation. Our research is at present centred on the generation of somatic variants.

Three major requirements for the successful application of somatic variation and other cell manipulation techniques in crop improvement are: a) an understanding of the nature and control of somatic variation; b) the existence of efficient and reliable methods for the regeneration of plants from culture, and for their rapid propagation; and c) the development of reliable methods for screening *in vitro* for novel characters. These areas are now being investigated using members of the genus *Brassica* as models.

The Brassicas are an economically important plant group, providing oilseed, fodder and vegetable varieties. Their genetics have been extensively studied, and the six major species have been shown to be genetically interlinked (U, 1935), giving three diploids *Brassica oleracea*, (n=9), *B. nigra*, (n=8), *B. campestris*, (n=10), and three amphidiploids, *B. carinata*, (n=17), *B.juncea*, (n=18), and *B. napus*, (n=19). Brassicas respond well to growth in culture, and have already been widely studied in this respect (Ingram 1983; MacDonald & Ingram 1984).

Fast flowering lines which flower within 19 - 21 days have also been developed (Williams 1982). These lines allow several generations to be grown a year in contrast to one or possibly two each year in the commercial brassicas. Research relevant to studies of somatic variation in brassicas is outlined below.

SECONDARY EMBRYOGENESIS

A central procedure in our work on the production of somatic variants is the generation of somatic secondary embryoids from Brassica tissues grown in culture. These are asexual embryoids which arise from the surface of tissues derived from anther culture, (Thomas et al. 1976; Loh & Ingram, 1982; Ingram et al. 1984). They have been shown to arise from single epidermal cells, and their development is similar to that of sexual embryos, passing through globular, heart and torpedo stages.

Anther culture techniques have been widely used to generate haploid and amphihaploid plant material in many species. In our research anther embryoids are obtained from winter rape varieties using the techniques of Loh & Ingram (1982), (See Newsholme et al this volume). Anther embryoids are transferred to MS medium (Murashige & Skoog 1962) and after 4 - 6 weeks 40 - 80% of the embryoids develop abnormally and give rise to secondary embryoids. These secondary embryoids may occur randomly or in lines over the surface of the hypocotyl and other tissues of abnormal plantlets. They are loosely attached to the parental tissue, and can easily be removed and transferred to fresh culture medium. A proportion of these in turn will give rise to further secondary embryoids. The expression of secondary embryogenesis has been shown to be extremely variable from one generation to another, and it is also affected by physical and chemical components of the culture environment, including temperature, number of embryoids per plate, and sucrose concentrations, (Ingram et al. 1984). This could be due to the unstable expression of developmental genes.

The majority of secondary embryogenic lines obtained through anther culture are haploid. These lines may be diploidised or raised to higher ploidy levels by treating young embryoids with colchicine. The surviving tissues retain their secondary embryogenic potential and give rise to further secondary embryoids with stable ploidy levels (Loh & Ingram 1983).

The regeneration of plantlets from secondary embryoids may be triggered when required, by growing embryoids on MS medium supplemented with 10^{-4} M kinetin (6-furfurylaminopurine) for 4 weeks (Loh et al. 1983). Recent research (unpublished data), has shown that treatment with kinetin for only three days or less may be sufficient to trigger the response. Tissues are

then transferred to a medium without growth substances, or with low levels of IAA (1-indole acetic acid), or with raised levels of thiamine to stimulate rooting. Rooted plantlets may then be potted in compost. The treatment with high cytokinins reduces, but does not eliminate, secondary embryogenesis; this may influence the number of stems per plant following regeneration.

Lines of secondarily embryogenic tissues of some cultivars have been maintained for 4 years with stable ploidy levels, and these are now being used to study variation and to develop *in vitro* screening techniques.

VARIATION

It has been suggested that plant cells and tissues grown in culture exhibit considerable somatic variation (Larkin & Scowcroft 1981; Scowcroft *et al.* 1984: Orton 1984), and where beneficial,this variation may have important implications in plant breeding. The origins of somatic variation are poorly understood, but it is possible that the culture process itself frees individual cells from the stabilising influence of the tissues of the whole plant although the possibility of pre-existing variation cannot be ignored (Ingram 1983). In haploid systems, produced through anther or pollen culture, it is clear that both dominant and recessive somatic variant characters will be expressed, although in the amphihaploids some recessive genes in one chromosome set may be masked by dominant characters in the other.

It is essential to understand the origins and genetic basis of somatic variation in order to be able to enhance and control it. The enhancement of variation could lead to the more efficient generation of novel characters, for example disease resistance, which may be of use to the plant breeder. Its suppression would be an essential step in the development of clonal multiplication and rapid propagation techniques, and the development of artificial seed (Ingram 1983).

It is also important to determine whether selected variant characters are under genetic control, or arise as the result of growth in culture. Some characters, such as those controlled by major genes can be analysed through conventional genetic techniques but characters under complex genetic control may require techniques such as restriction fragment mapping to determine their basis.

Scowcroft *et al.* (1984) list a number of crops in which some somatic variant characters have been shown to be sexually transmitted. These include wheat, oats, rice, maize, tobacco and the brassicas. Preliminary investigations, (Hoffmann *et al.* 1982; Scowcroft *et al.* 1983; Ingram *et al.* 1984) of plants

regenerated from anther embryoids and secondary embryoids of B. napus have revealed variation in a number of important characters: a) the number of stems per plant, b) distinct flower morphology, c) times to flowering following vernalisation, d) levels of erucic acid and glucosinolates, e) differences in the waxiness of leaves, and f) levels of resistance to disease.

SCREENING IN VITRO FOR DISEASE RESISTANCE

In order to exploit somatic variation efficiently, it is necessary to be able to screen the tissues in vitro. In the case of resistance to disease, there have been two main lines of approach. Firstly, the selection of tissues which exhibit resistance to pathogens in culture and secondly, the selection of tissues which exhibit resistance to pathotoxins incorporated into tissue culture media.

Screening with pathogens

The possibility of challenging plant tissue cultures with specific pathogens to select resistant lines has, to date, been insufficiently researched. There have been cases (Ingram 1977) where the response of tissues grown in culture have differed from those of the donor plants. Helgeson et al. (1976) however, have shown that in Nicotiana, major genes for resistance to race 0 of Phytophthora parasitica can be expressed in tobacco tissue culture systems, if the cultural conditions are carefully controlled. The balance of cytokinin to auxin in the culture medium was especially important in controlling the level of resistance expressed (Helgeson 1980).

Resistance to pathotoxins

Pathotoxins have been used successfully to screen tissue culture lines for resistance to a number of different diseases, where insensitivity of cultured cells to the toxins has been correlated with increased levels of resistance to specific pathogens in regenerated plants. Examples include maize and Helminthosporium maydis (Brettell et al. 1980); potato and Phytophthora infestans (Behnke 1979, 1980); alfalfa and Fusarium oxysporium f.sp. medicaginis (Hartman et al. 1984); and oilseed rape and Phoma lingam (Sacristan 1982). In each case, toxic culture filtrates or partially purified pathotoxins have been added to the tissue culture medium and tissues able to grow in their presence have given rise to plants resistant to the specific pathogens. Despite these successes, several basic conditions must be satisfied before such techniques can be used effectively. For each host-pathogen combination it must be ascertained that any toxins produced by the pathogen in culture are also produced in the infected host plant, and that the toxin(s) are implicated in the development of disease symptoms. It is also essential that if more than one toxin is produced, all are used in the screening procedures. At present far too

little is known of the role of pathotoxins in plant disease.

Screening for resistance in Brassicas

Loh (1982) screened plants from clonal secondary embryoids of the winter rape cultivar Primor for resistance to L. maculans (P. lingam) and showed that they were considerably more susceptible than in a seedling population of the same cultivar. He then incorporated Sirodesmin PL into the culture medium and grew secondary embryoids on this medium for one month. Surviving embryoids (10%) were plated on plain MS medium for three weeks to allow the development of further secondary embryoids. When these were transferred to a medium containing Sirodesmin a higher proportion of embryoids (30%) survived. Unfortunately, no information was obtained about the sensitivity of regenerated plants to the toxin or the pathogen. The slightly higher proportion of surviving embryoids rather than a uniform resistance could suggest that the increased resistance was an adaptation to the culture environment. Also, the precise role of Sirodesmin in canker has not been adequately determined, and it may only be a single component of the host-pathogen interaction.

Sacristan (1982) grew secondary embryoids of spring rape on a culture medium supplemented with toxic culture filtrates of L. maculans and regenerated plant from resistant lines. These showed enhanced resistance to the pathogen. She argued that the toxin of L.maculans causes necrosis on susceptible varieties and that the tissues are then colonised more rapidly by the fungus. Thus the non host-specific toxin was a co-determinant of pathogenicity, and insensitivity to this toxin caused the plants to be more resistant to the fungus. It is not clear whether the embryoids tested had been treated with mutagens, but preliminary analysis of the progenies suggested that the resistance was of a genetic nature. The single cell origin of secondary embryoids makes them suitable systems for mutagenesis to enhance the levels of variation. However, it must be remembered, that in most cases that have been genetically analysed, resistance to disease in plants is controlled by major genes (Ellingboe 1981) while many mutagens frequently cause deletions. This means that mutations affecting the expression of resistance, rather than the resistance genes themselves, would be required to create new disease resistant material (Ingram 1984). Sacristan's results could be an example of this.

The level of resistance of some oilseed rape cultivars to a number of pathogens such as L.maculans and Pyrenopeziza brassicae is relatively good. Resistance to others e.g. Alternaria spp. is poor, and there is no readily available source of resistance which could be introduced. It may be possible to use embryo rescue techniques to transfer resistance from some of the fodder turnips which show resistance at the leaf stage, but it is not yet known whether these varieties are

resistant at the pod stage (Newman 1984). This is of major importance in the oilseed rape crop, as most of the yield losses caused by Alternaria spp. are the result of the early shattering of pods. Another possible approach to the problem is to look for novel somatic variants carrying resistance among plants derived from tissue culture, if a suitable screening technique can be established.

Preliminary studies (unpublished data) with Alternaria brassicae and A. brassicicola suggest that, when grown on liquid medium in culture, both species produce toxic substances which, when applied to detached leaves, give necrosis and chlorosis similar to the host's reponse to the pathogen. These substances are non-host-specific, affecting tomato and hydrangea as well as the brassicas. When added to culture medium the pathotoxins caused the death of secondary embryoids of oilseed rape (see Newsholme et al this volume). The few surviving secondary embryoids were plated on plain MS medium, and the secondary embryoids from these tissues showed an increased resistance to the toxic medium. There is some evidence that a similar substance is present in leaves of B.napus infected with A. brassicicola. Experiments are now in progress to isolate and identify the substances, and to determine their role in infection. A number of regenerants which have survived growth on the toxic medium have grown on to flowering.

It should be possible to develop disease screens for other Brassica pathogens in culture. Some such as Sclerotinia sclerotiorum, are already known to produce toxins. Others, such as Pyrenopeziza brassicae, Albugo candida and Peronospora parasitica do not, but it may be possible to establish dual cultures of these. In the latter cases, however, it may be necessary to establish quantitative, rather than qualitative measurements of host-pathogen interactions to determine levels of resistance in these finely balanced systems (Helgeson 1983), as an imbalance in favour of one partner may result in the loss of the other (Ingram 1977).

In conclusion, the use of somatic variation may be regarded as traditional breeding in a test tube, and it may be only of short term benefit. In the longer term, progress must lie in the genome transformation techniques which are being developed in many laboratories. Reliable disease screening techniques, however will continue to be required in the assessment of new lines. The suppression of somatic variation could provide excellent opportunities for rapid clonal multiplication.

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MICROBIAL PESTICIDES: SELECTION AND GENETIC IMPROVEMENT

C.C. PAYNE, P. JARRETT

Glasshouse Crops Research Institute, Littlehampton, West Sussex, BN17 6LP, United Kingdom

ABSTRACT

The prospects of selection and genetic improvement of strains of bacterial, viral and fungal pathogens of insects are reviewed. Modern techniques of genetic manipulation should provide the means for developing useful new strains of these microbial pesticides through the recombination of characters governing pathogenicity for target insect pests, including virulence, speed of kill and improved host range.

INTRODUCTION

Natural populations of insects are regulated to some extent by microbial infections. Some of these pathogenic micro-organisms (including bacteria, fungi, viruses, protozoa and insect-parasitic nematodes) have been used both experimentally and commercially as pest control agents. However, the present use of such microbial pesticides is small by comparison with the use of chemical insecticides. Estimates from the US Environmental Protection Agency (EPA) suggest that annual sales of the 14 EPA-approved microbial pesticides amounted only to $10 million during 1982 in comparison with sales of $959 million on chemical insecticides (Klausner, 1984).

The main advantage of such biological control agents lies in their selectivity and hence in their ability to be used within integrated pest management systems. Such control programmes aim to preserve the natural enemies of pests by reducing chemical inputs. As chemical usage is lowered so the intense selection pressure for pest resistance to chemical pesticides is reduced and the useful life of a pesticide is prolonged. This is a major consideration at a time where pest resistance to chemicals continues to develop and new compounds become increasingly more difficult to isolate and more expensive to produce.

However, we cannot ignore the fact that many of the microbial pesticides available today fail to meet the expectations of growers attuned to the rapid and broad-spectrum pest-knockdown achieved by many chemical pesticides. There is a continuing research need to improve the efficacy of existing and future microbial pesticides. Perhaps the greatest defects of some commercial microbial products is that they have a very limited host spectrum of activity and are insufficiently virulent. There are mechanisms by which such factors can be improved. Wide differences in virulence between closely-related insect-pathogenic, fungal, bacterial and viral strains have been recorded and there is naturally-existing population variation from which the most appropriate pathogen strain can be selected. Further genetic variation can be induced by mutation, genetic manipulation and genetic engineering and it is these considerations that form the basis of this paper. Much research and commercial interest is now being shown in the strain improvement of insect pathogens and in the following sections we attempt to evaluate the importance of these studies and the prospects for a wider use of microbial pesticides.

STRAIN IMPROVEMENT OF MICROBIAL PESTICIDES

Bacteria

Strains of Bacillus thuringiensis have been commercially used in agriculture and horticulture as biological insecticides for several years with the first products from the commonly-used HD1 strain appearing in 1970. This bacterium produces a protein crystal (δ-endotoxin) at sporulation which, when ingested by susceptible larvae is degraded by proteolytic enzymes into a lethal toxin causing cessation of feeding, gross disruption of the gut and eventually death. Most of the B. thuringiensis strains isolated so far are pathogenic for certain lepidopteran larvae although strains have been identified (e.g. B. thuringiensis var israelensis) which produce crystals toxic to dipteran larvae including mosquitoes and blackflies (Goldberg & Margalit, 1977).

It has been known for some time that different lepidopteran pests differ in their response to distinct strains of B. thuringiensis (Dulmage, 1981) so that few naturally-occurring strains are highly pathogenic for the complexes of lepidopteran pests that occur on many crops. Reasons for this variation in host pathogenicity include the fact that the crystal protein varies between strains, some strains producing a mixture of distinct crystal proteins often providing a wider toxicity spectrum (Jarrett, unpublished observations). In addition, proteolysis of the crystal protein in the larval gut, essential for activation of the toxin, may differ between insect species and there is also the differential susceptibility of insects to the same toxin. Some strains (such as the commercially-used HD1 strain) have a relatively broad spectrum activity against a number of important pests, while other strains are more effective than HD1 against some pests but inactive against others (Table 1). Despite the fact that the biochemical basis of these differences is not yet understood, useful improvements in biological activity have been obtained by screening naturally occurring strains (Dulmage, 1981). Further improvements in potency have also been made by the production of new strains by mutation (Burges et al., 1984). Even greater improvements are likely from knowledge now being obtained on the genetic basis of strain potency.

TABLE 1

The relative activities* of four distinct B. thuringiensis strains against four noctuid pests

Insect pest	B. thuringiensis strain			
	a (HD1)	b	c	d
Heliothis virescens	42	2	100	52
H. armigera	90	6	100	49
Spodoptera littoralis	7.5	100	<1	24
Mamestra brassicae	15	100	2	32

* Activities expressed as relative potencies. The values are expressed as percentage activities of the highest potency (100) observed with each species (Jarrett, unpublished observations)

Recent studies on the genetic basis of crystal protein production have provided methods for the future development of improved B. thuringiensis strains. Suspicions that the gene(s) responsible for the production of the protein crystal was located on extrachromosomal DNA (plasmids) arose from the observation that B. thuringiensis strains in culture frequently lost the ability to produce crystals. The loss of certain plasmids was also noted in acrystalliferous mutants (Burges et al., 1984). Direct evidence of plasmid involvement in the commonly used B. thuringiensis var. kurstaki -HD1 Dipel strain came from the work of Schnepf & Whiteley (1981), who cloned plasmid DNA from this strain into Escherichia coli, obtained expression of the toxin activity and identified in the transformed bacteria a polypeptide antigenically related to the crystal protein and with a similar molecular weight (130,000). The production of gene-specific probes for the cloned crytal protein gene have shown that crystal protein genes are present in many B. thuringiensis strains on plasmids of varying size (Kronstad et al., 1983). The crystal protein gene(s) is sometimes present on more than one plasmid in a single strain and in some strains may be sited on the bacterial chromosome (Klier et al., 1982; Jarrett, 1983; Kronstad et al., 1983; Jarrett, unpublished observations). These studies suggested that the transfer of plasmids between strains could provide recombinants with improved potencies. Methods tested for DNA transfer between B. thuringiensis strains have included protoplast fusion and phage-transduction but these are technically more demanding than a newly-discovered conjugation-like mechanism (Gonzalez et al., 1982). Using this technique, plasmids can be exchanged between different parental strains to obtain recombinants (transcipients) some of which may possess the combined toxic properties of both parents. This is illustrated in Table 2, where transfer of a plasmid from strain A (with high potency for Heliothis armigera, but inactive against Spodoptera littoralis) to strain B (with low potency for H. armigera but moderate toxicity for S. littoralis) resulted in a transcipient combining the potencies of the two strains (Jarrett, unpublished observations).

TABLE 2

Activity of donor (strain A), recipient (strain B) and transcipient isolates of B. thuringiensis against S. littoralis and H. armigera

Strain	LC_{50} of B. thuringiensis strain (µg/g food)	
	S. littoralis	H. armigera
A	>10,000	48
B	298	598
Transcipient	220	39

Such studies make possible the tailoring of B. thuringiensis strains for improved toxicity and efficacy against a complex of lepidopteran pests

of a particular crop. Such manipulated strains could be patentable and provide commercial companies with a unique microbial pesticide in the market place.

Further studies on the precise nature of the toxin should be profitable. While the main toxic component derived from the crystal protein has an approximate molecular weight of 70,000 (Lilley et al., 1980), the nucleotide sequence of at least one crystal protein gene is known (Chang, 1983) and it should be possible to engineer smaller toxic proteins and modify the insecticidal specificity. Also, as the crystal protein is a single gene product, the cloning and expression of the toxin in higher plants is experimentally feasible and could lead to the development of crop plants capable of resisting caterpillar attack. Thus recent advances in molecular biology and genetic engineering provide a number of interesting possibilities for the improvement of pesticides based on B. thuringiensis.

Although B. thuringiensis remains the main target for bacterial strain improvement, other bacterial species such as B. sphaericus and B. popilliae produce toxins against mosquito larvae and Japanese beetle, respectively. Data on the genetic basis of their toxicity is still largely incomplete and further research is needed before major improvement in these products is possible.

Viruses

There are several major groups of viruses which cause disease in insects (Payne & Kelly, 1981). Of these, only baculoviruses have been given extensive consideration as microbial control agents (Payne, 1982). Within the baculovirus group, virus particles are comprised of rod-shaped nucleocapsids, (containing covalently closed circular DNA) surrounded by a unit membrane. In some members of the group the virus particles are packaged singly (granulosis viruses) or in large numbers (nuclear polyhedrosis viruses (NPVs)) within large proteinaceous inclusions late in infection. The main component of these inclusions is a matrix protein (referred to as 'polyhedrin' in NPVs) containing a single polypeptide with a molecular weight of about 30,000.

Baculovirus infections are common amongst Lepidoptera and Hymenoptera. Larvae become infected when eating food contaminated with the virus-containing inclusions. The matrix protein (polyhedrin) dissolves in the insect gut, releasing virus particles which infect and multiply in gut epithelial cells. In Lepidoptera the infection quickly spreads to other tissues, though the larvae are only killed three or more days after infection. One limitation therefore in the use of viruses as pesticides is their slow rate of kill. Unlike B. thuringiensis, baculovirus infections rarely cause rapid cessation of feeding and pest larvae may be able to cause considerable crop damage before death. However, spread of infection occurs much more effectively in insect virus infections than with B. thuringiensis because of the huge quantities of often highly infectious virus inclusion bodies (up to 10^{10} per larva in some NPV infections) that are released when the insect dies.

Many baculoviruses are highly host-specific and may seem potentially ideal candidates for selective pest control within integrated pest management systems. Several isolates have been successfully used on an industrial scale, predominantly in north America, particularly against forest pests (Payne, 1982). However, the high specificity of many baculo-

viruses is often disadvantageous in most cropping systems where a variety of lepidopterous pests (leaving aside other pest orders) may need to be controlled. Thus, although a granulosis virus can be highly effective in controlling codling moth, Cydia pomonella (Glen & Payne, 1984) it is necessary to find different selective methods of control for other lepidopterous pests (e.g. leafrollers) within the orchard. Although some baculoviruses appear to have an extensive host range (e.g. a NPV isolated from the alfalfa looper, Autographa californica has been reported as capable of infecting at least 40 lepidopterous species), the pathogenicity of the virus may still be inadequate to control the spectrum of pests on a particular crop. Comparing the infectivity of NPVs from A. californica and H. armigera for major cotton pests (Table 3) it can be seen that H. armigera NPV is likely to prove more effective but still has low infectivity for certain major pests e.g. S. littoralis.

TABLE 3

The relative infectivities* of two baculoviruses for neonate larvae of major lepidopterous pests of cotton

Pest	Virus	
	H. armigera NPV (Polish isolate)	A. californica NPV
Heliothis virescens	17[a]	100[b]
H. armigera	100[a]	< 5[b]
H. zea	100[b]	< 5[b]
Pectinophora gossypiella	8[b]	10[b]
Spodoptera littoralis	0.8[a]	6[b]

* Relative infectivities were calculated from LD_{50} (a) or LC_{50} (b) assays. The values are expressed as percentages of the highest infectivity (100) observed with each virus (C.C. Payne & C.F. Williams, unpublished observations).

Undoubtedly, improvements in baculovirus pathogenicity can be obtained by selection. Recent studies have also shown that genetic recombination occurs between closely related baculoviruses in mixed infections, providing a means of generating new variants (Croizier & Quiot, 1981). Perhaps the most exciting developments have come from recent studies on baculovirus genetic engineering which illustrate the possibility of introducing new genes into precise positions in baculovirus genomes. Although present studies have not been directed to improving baculovirus host range spectra or speed of kill, it can be envisaged that such modifications will become possible in the future.

In the most recent studies, Smith et al. (1983) and Pennock et al. (1984) have shown that A. californica NPV (AcNPV) can be used as a vector for the propagation and expression of introduced (passenger) genes. In

these studies genes coding for human beta interferon, or E. coli β-galactosidase were incorporated into the virus DNA by constructing plasmids containing the passenger gene inserted into the cloned polyhedrin coding region of the AcNPV DNA. Insect cells were co-transfected with the recombinant plasmid DNA and wild type AcNPV genomic DNA. Recombinant viruses were then isolated which expressed to a very high level in insect cells the products of the passenger genes i.e. interferon or β-galactosidase. The high level of gene expression (much higher than most other vector gene expression systems tested) can be accounted for by the high activity of the polyhedrin promoter as the polyhedrin gene is normally abundantly expressed during infection. This work illustrates, on the one hand, that insect viruses in cell culture may provide ideal eucaryotic vectors for expressing products from cloned genes. On the other hand, and more appropriate in the context of this paper, it also illustrates that such baculoviruses can both accommodate and express additional DNA sequences and suggests that new and infectious baculovirus strains could be constructed.

As in many areas of genetic engineering we need a better understanding of the physiological processes involved - in this case to understand the factors influencing baculovirus host range - before we can select those genetic characters which should be combined to provide a recombinant virus with the desired characteristics of host spectrum and speed of kill.

Fungi

Insect pathogenic fungi infect their hosts by penetration through the cuticle of the insect. This mode of infection clearly differs from that of the insect-pathogenic bacteria and viruses. As a result, the host spectrum of fungi is wider as they do not have to be ingested and therefore will also infect insects (e.g. aphids) which feed on untreated internal plant tissues. Although the high humidities required for spore germination often restrict the potential use of fungal pathogens, commercially-produced deuteromycete strains have been used for controlling pests within the Hemiptera and Acarina (Hall, 1981; McCoy, 1981). Nonetheless, as with both bacteria and viruses, important traits for biological control are found separately in different fungal isolates. Variation in virulence among strains of fungi has often been observed (Heale, 1982). The control of virulence is likely to be complex and concerned with rapid spore germination, regulation of enzymes such as chitinases and proteases required to penetrate the insect cuticle, as well as responses to humidity and temperatures.

Although hypervirulent fungal strains can be isolated from natural populations of fungi, the attempted recombination of selected traits between different strains using techniques developed in studies of the fungal parasexual cycle is a particularly interesting approach with deuteromycete entomopathogenic fungi (Heale, 1982). Using auxotrophic and conidial colour mutants of Metarhizium anisopliae, Messias & Azevedo (1980) observed heterokaryon formation between strains, and isolated presumptive diploids on minimal media sharing the characters of both parental strains. It seems likely that the parasexual cycle also operates in other entomopathogenic deuteromycetes and could be an important mechanism in producing new strains. Protoplast fusion as an improved means of generating heterokaryons looks encouraging though the subsequent stability of any hybrid strains needs further investigation. Nonetheless, such studies could be most fruitful; for example the potential combination of the 'whitefly'- and 'aphid'-pathogenic strains of Verticillium lecanii (Hall, 1981) could lead

to a hybrid fungal strain with the high spore-producing capability of the aphid isolate and the faster-germinating potential and higher pathogenicity of the whitefly strain (R.A. Hall, personal communication). Genetic characteristics which might improve spore germination at lower humidities would also be useful features to incorporate as this could extend the environmental range in which these pathogens could be used.

CONCLUSIONS

In such an article it is impossible to do more than highlight some of the genetic prospects of strain improvement in microbial pathogens. There is a clear need to improve many existing strains particularly in providing more desirable host spectrum activities for practical pest control. However, real genetic improvements are likely to be made in the next few years. New improved strains of B. thuringiensis have already been produced. As understanding improves of the factors governing the host pathogenicity spectrum of baculoviruses and entomopathogenic fungi, so it should be possible to select those genes which are required in useful recombinant strains. However, even when these strains are produced, regulatory clearance for the practical use of such engineered micro-organisms remains a major hurdle. At present the precise registration details of how such recombinant micro-organisms wil be treated have yet to be worked out (Klausner, 1984). Finally, the successful practical use of these agents is still dependent upon adequate methods of formulation and application to ensure that the pathogens are applied to the right target at the right time. The suggestion that biological control agents could, by the year 2000, occupy up to half of the insecticide market is probably over-optimistic (Klausner, 1984) but rapid advances in strain improvement should ensure a much larger future market share for microbial pesticides than the present one per cent.

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