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Obligate parasitism

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Introduction

In preparing my Presidential Address, I was torn between a desire to present a philosophically oriented talk on the one hand, and to present results of our more recent research work on the other. Previous addresses have shown no set pattern, some have been on wide issues relating to the science of Plant Pathology at the time, others have concentrated on their own particular research.

The Society membership cuts across a wide range of interests. At one end of the spectrum we have field workers and at the other end members making administration and policy decisions and scientists using the latest technology.

In an attempt to give an address in which at least parts will be of interest to all members of the Society, I plan to present what I see as some of the important issues facing plant pathologists today; and discuss in a general way some of the results of the research of my laboratory during the last few years.

Important issues currently facing plant pathologists

Erosion of the genetic variability of crops—This is brought about by the cultivation of a few major varieties with the perceived favorable phenotypic traits determined by market pressures. This has resulted in the development of fewer cultivars which have a very narrow genetic base for resistance to disease and other stresses. Two basic breeding approaches have been advanced to combat the dangers of genetic uniformity. The first is breeding to eliminate defects as soon as they appear; the weakness of this approach is the time it takes for plant breeders to respond to anticipated threats. Even the incorporation of a new gene for resistance takes more than 5 years in most crops and in some as long as 20 years. In the future this time barrier may be overcome using recombinant DNA technology. The second approach is the deliberate introduction and retention of variability in crop populations with the object of ensuring that part of the population will survive every crisis. Both methods, and indeed all breeding, depend heavily on germ plasm resources. Unfortunately, primitive and wild cultivars which have formed the basis of genetic variability in the past are being destroyed by the encroachment of man on nature, and exotic germ plasms are a rapidly receding resource.

Pollution of the environment with agrochemicals—This problem is particularly acute in central Europe and North America; the point has now been reached where crop production is totally dependent on the use of these chemicals to control weeds and other pests such as viruses, bacteria, fungi and insects. Each year there is an increasing use of pesticides and, at the same time, there is an increasing amount of damage caused by pests but, without the use of chemicals, crop destruction would be complete. This has resulted from a largely empirical approach to the development of chemical controls in which, with little or no prior knowledge of their mode of action, the chemicals are selected on the basis that they kill the target pest and do minimum damage to the crops and/or the environment. The net result is that the target pest develops resistance to the chemicals and in the meantime damage is done to the environment. What is missing is a basic understanding of both predator and host.

Problems presented by biotechnology—The last decade has seen a tremendous increase in our understanding of genes and how these can be manipulated in transformation experiments. However, there has been a tendency to overstate the role that recombinant technology will play in plant production in the immediate future. At this stage, with rare exceptions, we do not have agriculturally useful genes at our disposal and, in addition, the most important of agricultural species, namely the cereals, are not amenable to transformation experiments. It is patently obvious that many years of hard research will be required before these two obstacles will be removed. This type of research is very costly and the cost is exacerbated when one relates this type of work to plant pathology laboratories which are usually not equipped to carry out basic biochemical research. This problem is also confronted when training plant pathologists for a future in which it will be important for some of them to be able to relate recombinant DNA technology to plant disease problems. This increasing-cost situation arrives at a time when governments are not really in tune to giving increases in financial support to plant sciences because in the developed countries we already have an over-production of plant products.

A 'down the track' problem that plant pathologists will encounter with genetically engineered organisms is public opposition to their deliberate release

of such organisms into the environment. This has already been witnessed in the U.S. in the release of genetically engineered epiphytic bacteria designed to protect plants against frost damage.

What we as plant pathologists should be doing to help agricultural production in Third World countries—The Third World agricultural production needs help. Although Asia and Latin America have experienced a mini green revolution over the last two decades, food production in these regions is still struggling to stay ahead of population increases. The poorer people are still hungry and suffer from malnutrition. The situation in Africa is the most critical; that continent is just emerging from its worst drought in 50 years where, at best, the rainfall is low and unpredictable. Soil quality is poor, traditional farming systems are inadequate, and the continent seems to harbour its own peculiar plant and animal diseases and insect pests unheard of anywhere else. Given the growing population in these Third World countries and the current agricultural practices, attempts to feed themselves are leading to very serious erosion of the natural resources base. In October 1986, the National Academy of Sciences and the Smithsonian Institute sponsored a national forum on what was perceived to be a global crisis — the potential imminent extinction of perhaps half of the world's species, mainly through deforestation. The forum concentrated on the tropical moist forests of the Third World countries because these are the richest in species of all major habitats, and because they are in the greatest danger. These forests cover about 7% of the land surface of the globe but contain 50% of the species. The clearing of land brought about by population pressures and the increasing necessity to produce greater quantities of food is considered to be a major crisis. The challenge we face as plant pathologists is to educate Third World people in ways of controlling plant diseases and protecting their crops so that their use of existing land is made more efficient.

Research in my laboratory

I would now like to spend the remainder of my talk highlighting results of our investigations on obligate parasitism by phytopathogenic fungi.

Phytopathogenic fungi, which are strict obligate parasites, are those species which grow and reproduce in nature only in association with living host plants and cannot be grown in axenic culture, i.e. in the absence of living cells of other species. Obligate parasitism is found in both non-filamentous and filamentous groups of fungi. Well known examples of non-filamentous obligate parasites attacking higher plants and causing economically important diseases are the intracellular fungi *Plasmodiophora brassicae* (club root of crucifers) and *Spongospora subterranea* (powdery scab of potato). In the filamentous fungi, obligate parasitism occurs in the three major classes. In the Phycomycetes, members of the families Peronosporaceae (downy mildews) and the Albuginaceae (white blisters), comprising about 140 species and causing great economic losses in cultivated plants, are classified as obligate parasites. Members of the Order Erysiphales of the Class Ascomycetes, comprising about 90 species causing powdery mildew disease in a wide range of crops, are considered to be obligate parasites. With a few exceptions the whole of the Order Uredinales, comprising about 4500 species in the Class Basidiomycetes, are classified as obligate parasites. The Uredinales, known as the rusts because of the reddish brown colour of some of the spore forms, possibly cause greater crop loss than any other group of fungal pathogens.

Three intriguing aspects of the study of obligate parasitism can be discerned. These are

(i) the growth of these groups of parasites in axenic culture;
(ii) their host specificity, which in some instances is extreme and the unique relationship which exists between host and pathogen where successful parasitism is established is akin almost to symbiosis rather than parasitism;
(iii) the elucidation of molecular mechanisms determining resistance to these pathogens.

Culture of obligate parasites—In 1966 we reported the first successful culture of a rust fungus from uredospores. In hindsight the three prime reasons for our success were: firstly, the use of aseptic conditions allowing for long periods of incubation of experimental material; secondly, monitoring sporeling development not as a function of rate of elongation of germ-tubes but rather using branching and septa formation as indices of growth; and thirdly, working in a broadly based department of Biochemistry allowed access to a wide range of techniques and materials.

The necessity of using aseptic conditions was emphasized by comparing the rates of elongation of germ-tubes from the uredospores with growth rates of intercellular hyphae in susceptible hosts. Rates of germ-tube elongation can be as high as 75 μm h^{-1}. In contrast, the rate of growth of intercellular hyphae in susceptible hosts is approximately 5–10 μm h^{-1}. It was anticipated that growth rates on artificial media would be slow and probably slower than that of intercellular hyphae. Thus, extended periods of incubation would be necessary for significant saprophytic growth to occur, and this required the use of aseptic techniques.

Uredospores free of contaminating microorganisms were obtained by a method of aseptic leaf culture and incubated on nutrient medium containing Czapek's minerals, 0.1% yeast extract, 0.1% Evan's peptone and 3% sucrose. Septa formation and continued branched growth over extended

periods of incubation were used as an index of growth rather than germ-tube length over short periods of incubation.

In tracing the development of the mycelium it was observed that germination and germ-tube elongation had occurred after two days. Initial branching of germ-tubes was observed 4 days after inoculation and this branching continued for 3–4 weeks. Some cultures formed stromata by curling up from the agar surface and stromatic tissue bore both uredospores and teliospores. The pathogenicity of primary cultures has been demonstrated using both mycelium and uredospores.

In the original experiments on culture of the wheat stem rust fungus, Evan's peptone was required in the medium. This is a proteolytic digest of whale muscle and one can raise the intriguing question as to why such a product should support the growth of a parasite of cereals. In experiments designed to determine the active principles in peptone, it was shown that, in addition to a source of nitrogen, sulphur amino acids were required for growth. In an attempt to explain this nutritional requirement for a sulphur amino acid, axenic cultures were grown in the presence of ^{35}-S sulphate and ^{35}-S sulphide and the incorporation of label into sulphur amino acids and proteins was determined. The results obtained showed that ^{35}-S sulphide, but not ^{35}-S sulphate, was incorporated into organic forms. These results suggest that a metabolic block exists in the pathway of inorganic sulphur metabolism. Possible locations of such a block could be sulphate uptake, sulphate activation, or sulphate reduction. Sulphate uptake was shown to occur at a rate comparable to other fungi thus eliminating this possibility. A block at sulphate activation also appears unlikely, since the end product of these reactions, adenosine 3'-phosphate 5'-phosphosulphate (PAPS) is required by the fungus for the synthesis of sulphate esters as well as sulphur amino acids. This would be a lethal block and no growth of the fungus would occur at all. It appears more likely, therefore, that this fungus is unable to reduce PAPS to thiosulphate or sulphide.

Of rust fungi so far investigated, all require the presence of sulphur amino acids when grown on a chemically defined medium. This nutritional requirement is of particular interest because sulphate reduction is found in prokaryotes such as bacteria and blue green algae; eukaryotic algae and most fungi can reduce sulphate and will grow on sulphate as the sole sulphur source. Sulphate reduction occurs in all higher plants so far tested.

The host-pathogen specificity—The basic principle of parasitism is one of nutrition involving two living organisms, one the donor and the other the receiver of metabolites. It may be argued that the ability of an obligate parasite to establish a successful relationship is dependent upon its gaining access to host cells and creating a suitable metabolic environment within the cells. We have used the barley/powdery mildew system as a model for studying host–pathogen specificity. This system has three advantages: its ease of handling, a good basic understanding exists for pathogen development, and the availability of a number of near isogenic cultivars differing in resistance genes to the powdery mildew fungus. Using the chloroplast as an example, I want to illustrate the delicate balance of parasitism exhibited by this group of fungal pathogens. Judging by the reaction of chloroplasts to fungal infection there are two populations of chloroplasts in barley leaf cells.

(1) Chloroplasts that are susceptible to presence of fungus and subject to accelerated senescence during infection by the fungus.
(2) Chloroplasts that appear to be completely unaffected by the presence of the pathogen, and, in the instance of 'green islands', photosynthetic activity appears to be enhanced.

We have monitored the amount of accelerated senescence in chloroplasts susceptible to the presence of the fungus by measurements of overall photosynthetic rates and of components of the electron transport chain and activities of stromal enzymes. Commencing at 48 h after inoculation there is a marked decline in photosynthesis as measured by oxygen evolution. This is mirrored by a decline in chlorophyll content and aldolase activity. In attempts to identify rate-limiting steps in photosynthesis which are initial targets for fungal infection, we have measured the activities of combined photosystems 1 and 2, photosystem 2 and photosystem 1 individually, as well as the concentrations of cyt f, cyt b559, P700, and total and photoreducible plastoquinone. Activities of stromal enzymes have also been monitored over the course of infection. The conclusion drawn from the results of these measurements is that it is not possible to recognize a prime single target in the electron transport chain or CO_2 fixation pathway but that all components decline at the same rate.

Measurements of the amount of plastoglobuli (osmiophilic granules which accumulate lipids from adjacent thylakoid membrane breakdown) reveals that their concentration is much higher in infected than control leaves. This would support the hypothesis that certain populations of chloroplasts are subjected to autonomous *in situ* degradation of chloroplast constituents, but the double outer membrane remains intact. One is now confronted with the question as to the mechanism invoked by the fungus to produce this result.

A partial understanding of the basis of this phenomenon has been obtained by studies on *in vivo* and *in vitro* protein synthesis and on relative amounts of chloroplast and cytoplasmic polysomes in infected tissues. The rate of *in vivo* and *in vitro* protein synthesis is decreased in infected leaves compared

to control leaves of the same age. The levels of mRNA coding for the large and small subunits of ribulose-bisphosphate carboxylase is also decreased after infection. It has further been shown that chloroplast polysome content of susceptible barley leaves is decreased after infection. It appears that this decrease is due to breakdown of chloroplast mRNA by a specific RNAase. This is evidenced by an increase in mRNA-fragment-bound chloroplast monosomes *in vivo*, and increases in monosome content of chloroplast polysome preparations treated *in vitro* with RNAase isolated from infected susceptible leaves.

Our working hypothesis is that the fungus triggers the synthesis of a host RNAase which specifically degrades mRNA in some chloroplast populations but not others. This allows for an increased release of amino acids for fungal nutrition, and possibly other metabolites, and at the same time photosynthesis in the unaffected chloroplasts to proceed normally. A full explanation of these events may be forthcoming by applying the new tools of molecular biology.

Factors determining resistance—'One of the most challenging problems facing Plant Pathologists today is the elucidation of mechanisms which determine whether a plant is resistant to a particular pathogen.' This statement is taken from a review I wrote in 1972 and it is equally true today. It highlights the lack of progress which has been made in unravelling the molecular basis of disease resistance despite the enormous advances made in our understanding of gene expression. Using near-isogenic cultivars of barley developed by Moseman we have conducted a series of experiments on powdery mildew infected susceptible and near isogenic resistant cultivars carrying the Mla, Mlp and Mlk genes for resistance. Our working hypothesis is that resistance gene expression is induced after infection. This results in the formation of new mRNAs which when translated using *in vivo* or *in vitro* translation systems synthesize proteins which are unique to infected resistant hosts. If this is true then it should be possible to make cDNA copies of these new mRNAs and use these to study the induction of mRNAs and also to screen genomic libraries of resistant hosts for resistance genes. Experiments to test this hypothesis are discussed below.

Protein synthesis: *In vivo* protein synthesis was compared in non-infected and infected near-isogenic susceptible and resistant cultivars carrying the Mla, Mlk, and Mlp genes for resistance. ^{35}-S methionine was incorporated into leaf proteins of different experimental materials, the proteins extracted and separated by 2-dimensional electrophoresis. Analysis of 2-dimensional gels revealed two new groups of proteins. Firstly, several polypeptides showing enhanced labelling were detected in both susceptible and resistant hosts after infection but enhancement was always greater in the resistant leaves suggesting that the resistance loci have a regulatory function. Secondly, common resistance-related polypeptides appear in cultivars carrying the Mla, Mlk and Mlp genes at 24 and 48 h after inoculation. Changes in leaf mRNA populations of near-isogenic resistant and susceptible barley were investigated using *in vitro* translation of poly A$^+$ RNA followed by 2-dimensional electrophoresis. Analyses of gels showed that 15 mRNA species from infected leaves exhibited new or enhanced translational activity when compared to non-inoculated controls. Of these:

(i) 9 mRNAs were detected as early as 12 h following inoculation in both resistant and near-isogenic susceptible plants (infection-related mRNAs).
(ii) 6 mRNAs showed greater translational activity in leaves of infected plants carrying either the Mla or Mlp genes when compared to near-isogenic susceptible infected plants (resistance-related mRNAs).
(iii) 3 of the 6 mRNAs were common to both resistant cultivars.

Preparation of a cDNA library: A cDNA library was prepared from poly A$^+$ RNA isolated from infected leaves carrying the Mlp gene for resistance (cv.Mlp). The library was screened by differential hybridization using ^{32}-P labelled cDNA prepared from poly A$^+$ RNA of both control and infected leaves of cv.Mlp. Six cDNA clones showing greater hybridization to cDNA from infected leaves were selected for further characterization. The size of the cDNA inserts of these 6 clones ranged from 160 bp up to 820 bp. Using a northern blotting procedure it was possible to demonstrate that these cDNAs were of host and not fungal origin, and each cDNA hybridized to a single RNA species of different size. Thus, each of these 6 cDNA clones represents a distinct infection-related mRNA.

The kinetics of induction of the infection-related mRNAs were studied by hybridization of the cDNA inserts to total leaf RNA from control and inoculated leaves of cv.Mlp and cv.mlp. Only a small amount of hybridization of the cDNA clones to RNA from leaves of non-inoculated controls was observed with no differences between RNA from resistant and susceptible controls. These background levels of hybridization did not alter during the ageing of control leaves. During the 2 d infection period studied, the mRNAs corresponding to clones 2, 3, 5 and 6, were induced to a much greater extent in cv.Mlp when compared to that of cv.mlp, ranging from 2.8- to 5.7-fold depending on the cDNA clone used for detection. Similar results were obtained when these hybridization studies were extended to cultivars carrying the Mla and Mlk genes for resistance.

These results are consistent with the model which proposes that the resistance gene functions in a regulatory role after interaction with a race specific ligand of the pathogen.

Our aim is now to obtain genomic clones corresponding to these cDNAs. These genomic clones will allow an investigation of the primary sequence and the regulation of expression of these genes coding for resistance-related mRNAs. This should provide further insight into the molecular basis of plant resistance to obligate pathogens.

In conclusion, I would like to thank you for allowing me the honour of being President of your Society for the past 2 years. Also I would like to acknowledge a very rewarding collaboration with colleagues and students over the past twenty years.