Invited Trends Article

Plant Resistance Genes for Fungal Pathogens – Physiological Models and Identification in Cereal Crops

Wolfgang Knogge

Max-Planck-Institut für Züchtungsforschung, Abt. Biochemie, Carl-von Linné-Weg 10, D-5000 Köln 30, Bundesrepublik Deutschland

Z. Naturforsch. **46c**, 969–981 (1991); received July 22, 1991

The complex biological phenomenon "resistance" can be reduced to single Mendelian traits acting on both the plant and the pathogen side in a number of pathosystems. According to the "gene-for-gene hypothesis", the outcome of a plant/pathogen interaction in these cases is incompatibility if a plant carrying a particular resistance gene and a pathogen with the complementary avirulence gene meet. This suggests a causal role of resistance genes in a recognition process initiating active plant defense responses. Fundamentally different strategies are followed to identify these genes molecularly depending on the plant and pathogen species involved. Fungal diseases of crop plants, especially those of cereals, cause dramatic yield losses worldwide. It is assumed that a molecular characterization of plant genes conferring resistance to fungal pathogens will lead to a better understanding of the plant defense system in general permitting the development of new methods of crop plant protection.

General principles: animal *versus* plant defense

Plants, like animals, have evolved active mechanisms to resist disease-causing microorganisms. These defense systems must meet two basic requirements: the differentiation between "non-self" and "self" (recognition system) and the triggering of protection responses (effector system). In the circulation-based immune system of vertebrates, both are achieved by the complex interaction of different types of specialized cells, the lymphocytes [1]. Recognition of "non-self" is effected by preexisting B lymphocyte clones through an exquisite diversity of antigen-binding specificities arising from different somatic diversification processes [2]. The genetic information encoding immunoglobulin polypeptide chains is contained in multiple gene segments. Somatic recombination occurs during lymphocyte development leading to the formation of complete genes through the assembly of individual segments. Additionally, the sequences conferring immunoglobulin-binding specificity show a high rate of somatic mutation. As a consequence, each individual is essentially capable of recognizing any possible foreign ("non-self") compound. Upon pathogen invasion, those lymphocyte clones which fortuitously exhibit the appropriate specificity are activated and trigger the defense response. Recognition of "self" is effected by binding of the proteins of the major histocompatibility complex by the T cell receptor. These proteins are specific for an individual and play a role in the activation of lymphocytes.

In comparison with the vertebrate immune system, little is known about the active defense mechanisms in plants. In the absence of a circulatory system, no analogous mobile surveillance system evolved in plants. Instead, since each plant cell represents a potential target for pathogen attack, each must have both elements, recognition and effector systems.

A common and very successful defense effector system of plants is the rapid, localized cell death at infection sites (hypersensitive response), often accompanied by the synthesis of low molecular weight antimicrobial compounds, the phytoalexins [3, 4]. The chemical nature of these compounds appears to be species- or family-specific, whereas phytoalexin biosynthesis as such and the localized sacrifice of plant tissue represent more general principles [5–7]. Quite a number of reactions induced upon pathogen attack together with numerous pathogen- and plant-derived (endogenous) elicitors of these reactions have been identified during the last decade [8–16]. However, the actual functions of most of these pathogenesis-related plant responses remain unknown. In particular, the mechanisms underlying specific recognition of

Reprint requests to W. Knogge.

Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0939–5075/91/1100–0969 $ 01.30/0

invading microorganisms and signal transduction leading to the activation of plant defense genes are poorly understood.

A widely accepted hypothesis assumes that plant resistance exists at the species and at the cultivar level [17–19]. Each species is resistant to essentially all potential pathogen species (basic resistance). Few pathogens have developed mechanisms to successfully parasitize a particular plant species, thereby negating its basic defense (basic compatibility). The complex mechanisms leading to basic compatibility are controlled by pathogenicity genes. In fungal pathogens which actively penetrate plants, these genes may encode enzymes required for the penetration process and for the colonization of host tissue, suppressors of the plant defense response or cytotoxic compounds. Resistant cultivars originate from individuals of the plant species which, during further coevolution, regained resistance to some physiological races of the pathogen by random mutation. This cultivar-specific resistance is encoded by resistance genes. The hypothesis implies that cultivar-specific resistance is superimposed on basic compatibility.

Principles of recognition in plant defense

The phenomenon of self-incompatibility [20], a mechanism preventing self-fertilization in many plant species, demonstrates that plants were capable to evolve a "self-recognition" system in a different context. An analogous system may also operate in the defense process. It seems unlikely, however, that plants possess a system at the level of basic resistance capable of recognizing all potential pathogens in all cells but rather recognize more general phenomena such as disturbances of cell wall integrity. This could be achieved, for instance, by the deposition of "self" signal molecules (endogenous elicitors) in the cell wall [21–25]. Upon their release by wall-degrading pathogens, they may be recognized by plasma membrane receptors which trigger the defense response of the affected cells. Non-recognition in basic compatibility may reflect an evasion of the signal/receptor interaction, for instance by destruction of the signal or suppression of its binding. In cultivar-specific resistance, recognition might be based either on an inactivation of the pathogen avoidance mechanism or on the evolution of a separate or allelic endogenous elicitor/receptor system.

Alternatively, or in combination with the "self-recognition" of endogenous elicitors, plants may have evolved mechanisms to recognize common surface structures or secreted molecules of pathogen species or families (recognition of "non-self"). In this case, basic susceptibility may originate from structural variation or masking of these compounds by the pathogen or again from suppression of their binding. Cultivar-specific resistance would then be achieved through recognition of a different specific feature (race-specific elicitor) of a pathogen.

Both principles, recognition of "self" and "non-self", may be simultaneously valid for a given plant species and even lead to synergistic activation of the effector system [9, 11]. For example, recognition of "self" may determine basic resistance whereas cultivar-specific resistance may originate from recognition of "non-self". For true pathosystems, which represent the current state in plant/pathogen coevolution, the recognition mechanism may be different from that in basic resistance. In addition, the type of plant/pathogen interaction may have influenced the evolution of a particular mechanism and the origin and nature of resistance genes. While the strategy of biotrophic pathogens aims at the avoidance of the plant defense response necrotrophs tend to overpower it [26].

The significance of resistance genes in plant defense

The inheritance of resistance has been extensively studied in pathosystems with cultivar-specific resistance of the host. Genes conferring resistance to individual pathogen races have been defined in many plant species. In particular, in cereal plants, numerous genes encoding resistance to mildews, rusts and other fungal pathogens are known. The genetic basis of cultivar-specific resistance is best described by the gene-for-gene hypothesis: the outcome of a plant/pathogen interaction is determined by a pair of complementary genes – a resistance gene in the host plant and an avirulence gene in the pathogen [27, 28]. Unlike vertebrates, which do not have defined genes analogous to plant resistance genes, plant cultivars exhibit resistance to pathogen races carrying particular avi-

rulence genes only if they happened to inherit the complementary resistance genes. The pathogen product or function which is recognized by the plant (disease determinant) must be encoded by the avirulence gene. The plant product or function which determines recognition of particular pathogen races is encoded by the resistance gene. This gene must therefore be considered a sensor gene according to the Britten and Davidson hypothesis for gene regulation [29]. Depending on the type of disease determinant, the significance of resistance genes in recognition may be different. They are the basis of specific resistance in pathosystems with cultivar-specific elicitors. When the specificity of an interaction is based on suppressors of host defense or on host-specific toxins, however, resistance genes are the basis of unspecific resistance. In this situation, cultivar specificity lies in susceptibility.

Resistance genes are frequently dominant or codominant, but recessive resistance genes are known as well, such as the *ml-o* alleles in barley [30]. In diploid fungal species from the Oomycete and Basidiomycete families, avirulence genes are also frequently found to be dominantly inherited [31–34]. However, due to the haploid karyophase of the infectious vegetative stage of Ascomycetes and probably most imperfect fungi, dominance of avirulence or virulence is meaningless in the interaction of such pathogenic species with their host plants.

Although the gene-for-gene hypothesis was established in the early 1950s, no resistance gene has yet been isolated. Consequently, one of the most challenging goals of current plant biology is the molecular identification of these genes, the characterization of their immediate functions and their regulation.

General strategies for isolation of plant resistance genes

The approaches currently being used to identify plant resistance genes fall into two different categories (Fig. 1). The "structural" approach aims directly at the gene without prior knowledge of its product or immediate function [35, 36]. It involves identification of the gene's map position in the genome using molecular DNA probes and subsequent identification of the gene by a directed chromosome "walk" ("reversed genetics", map position-based cloning). Alternatively, the gene can be mutated by insertion of a molecularly characterized transposon followed by isolation and structural characterization of the tagged gene (transposon tagging) [35, 37].

The "functional" approach is based on the gene's product and its predicted immediate function. The first and crucial step is the identification and isolation of the complementary avirulence gene product from the pathogen. The resistance gene product can then be identified *via* its hypothesized interaction with the avirulence gene product or function [35]. The cloning of the resistance gene can consequently be achieved by conventional techniques. The approach for identifying a fun-

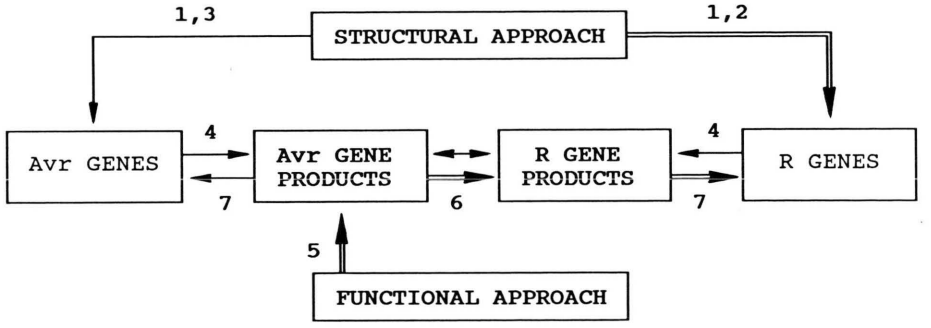

Fig. 1. Strategies for isolating plant resistance genes. The structural approach aiming directly at a gene without prior knowledge of its product comprises strategies such as map position-based cloning (1) and transposon tagging (2). The functional approach starts with the identification of the complementary avirulence (avr) gene product using genotype-specific resistance responses of the plant (5). The product of the resistance (R) gene can be identified through direct molecular interaction studies (6). R and avr genes can then be isolated by conventional cloning (7). In addition, bacterial avr genes have been identified by shotgun cloning (3).

gal avirulence gene and its product can again be "structural" or "functional". In addition to map position-based cloning, bacterial avirulence genes in particular, have been identified by shotgun cloning [38, 39]. A purification scheme can be developed to identify the avirulence gene product if resistance genotype-specific plant reactions such as the hypersensitive reponse or other differentially induced defense reactions have been characterized. The avirulence gene can then be cloned by conventional techniques. Expression of the avirulence gene in a heterologous organism such as *E. coli* or yeast should allow the production of quantitative amounts of the gene product. Irrespective of the strategy used, the resistance gene nature of the cloned gene should be finally clarified by complementation experiments in transgenic plants.

Cereal/fungus pathosystems as experimental models

A number of different pathosystems involving di- and monocotyledonous plants and viral, bacterial, fungal or nematode pathogens are presently being used to isolate plant resistance genes. The strategies described below are illustrated primarily using cereal plant species.

Most cereals meet several important requirements for approaches to isolate resistance genes: they are extensively analyzed genetically and chromosome maps as well as near-isogenic lines for numerous resistance genes are available. On the other hand, the additional prerequisites of transformation and regeneration are still in their infancy. In barley and wheat these methods are, as yet, far from routine although progress has been made with rice [40] and maize [41]. Recently, the first barley cultivars have been regenerated from protoplasts [42] and stable transformation has been achieved [43].

The fungal pathogens of cereals are genetically less well defined than their hosts. The rust fungi, for example, show very complex life cycles involving up to five different spore types [44, 45]. Furthermore, most of these basidiomycetes require alternate non-cereal hosts for the completion of their sexual cycles. The basidiospores of barley and wheat stem rust (*Puccinia graminis*), for instance, infect only *Berberis* spp. Similarly, barley leaf rust (*P. hordei*) requires *Ornithogalum umbellatum*, and common corn rust (*P. sorghi*) relies on *Oxalis* spp. As a consequence, crossing experiments are difficult to perform. For members of the imperfect fungi such as *Pseudocercosporella herpotrichoides* (wheat and barley eyespot) or *Rhynchosporium secalis* (barley leaf scald), the lack of a perfect (sexual) stage prevents crossing experiments. Gene transfer has been successful, although at low frequencies, with several pathogenic fungi of dicotyledonous plants [46–50]. However, the rice blast fungus, *Magnaporthe grisea* [51], and the maize pathogen *Cochliobolus heterostrophus* [52] so far remain the only examples of successfully transformed fungal pathogens of cereal species. The transformation of biotrophic fungi such as powdery mildews (*Erysiphe graminis* spp.) and rusts requires the development of new techniques since they cannot be cultivated outside their host plants. It is, therefore, difficult to carry out biochemical studies with these fungi (functional approach).

The structural approach

Transformation of a susceptible plant cultivar with a dominant resistance gene should result in the acquisition of resistance [35]. Therefore the random cloning of genomic DNA from a resistant cultivar and its transfer into a susceptible cultivar (shotgun cloning) should produce resistant transformants. But even under the assumption that cereal plants could be easily transformed and regenerated, a number of problems restrict the use of this strategy in cloning resistance genes from plants in general and from cereal plants in particular. Due to haploid genome sizes ranging from 0.6×10^9 bp for rice to 17.3×10^9 bp for wheat [53], 100,000 to millions of transformants must be produced and screened to identify a single gene. An additional complication arises from the ploidy level of species such as wheat, many cultivars of which are tetra- or hexaploid. Strategies are, therefore, being developed to reduce the number of clones necessary for transformants to be screened.

Map position-based cloning

Mapped resistance genes which are tightly linked to characterized loci for which cDNA or genomic clones are available are amenable to chromosome "walking" strategies. An example is the *Ml-a* locus of barley, which is almost equidistant

from two structural hordein genes, *Hor-1* and *Hor-2* [54]. The distance between these genes is about 8.5 cM. However, cases such as this one are rare. An alternative is the identification of flanking DNA restriction fragment length polymorphisms (RFLPs). RFLPs are caused by specific differences in the DNA sequences of individuals within a plant species which abolish or create cleavage sites for restriction endonucleases. The resulting differences in DNA fragment sizes can be detected with suitable probes [55] which can be generated in essentially unlimited numbers. They can be random, cloned single or low copy genomic fragments, cDNAs or characterized cloned genes. Since RFLP markers act as codominant Mendelian traits, their inheritance can be followed in crossing experiments. RFLPs are presently being exploited to develop detailed linkage maps not only of a number of dicotyledonous species such as tomato [56, 57], potato [58], lettuce [59], soybean [60] and *Arabidopsis thaliana* [61, 62] but also of monocotyledonous species such as maize [63, 64], rice [65] and barley [66].

Once a linkage map is established, any given genetic trait which can be screened at the phenotypic level, including resistance, can be mapped relative to the RFLPs by segregation analysis. The goal is to identify RFLPs which are linked to the target gene on both sides as close as possible. These RFLP markers serve subsequently as starting points for a chromosome "walk" to the target.

Such a strategy has been successfully applied to the mapping of maize gene *Mdm1* conferring resistance to maize dwarf mosaic virus [67]. Since it was known that this gene is closely linked to the yellow endosperm locus, *Y1*, on chromosome 6, DNA probes were chosen from the established maize RFLP map which are located in the vicinity of *Y1*. The positions of 4 RFLP markers were established relative to *Mdm1* and *Y1*. It was found, furthermore, that the resistance locus was surrounded by two closely linked RFLP marker loci which now can be used to "walk" to the gene.

The construction of an extensive and detailed RFLP linkage map of a given plant species requires the mapping of hundreds of clones, only a few of which will be tightly linked to the gene of interest. The availability of pairs of near-isogenic lines differing only in the vicinity of the target locus can therefore significantly speed up the identification of linked markers [68]. Many crop cultivars originate from backcrossing programs by which a gene of interest, such as a specific resistance gene, was introgressed into an agronomically valuable cultivar. It is assumed that after a number of backcrosses under selection for the desired phenotype, the genomes of the recipient cultivar (recurrent parent) and the backcross products are nearly identical (near-isogenic lines). The genomes of the backcross lines contain, however, donor-derived chromosome segments of varying size including the gene of interest (introgressed segment) [69]. This phenomenon can be exploited to quickly identify genomic clones which are tightly linked to the target gene. Only clones located within the introgressed fragment exhibit RFLPs between the near-isogenic lines and the recurrent parent whereas clones located outside will display identical restriction fragment patterns.

An important prerequisite for the identification of RFLPs using this strategy is sufficient genetic divergence between the donor and recurrent lines [70]. The more divergent the DNA sequence of the introgressed chromosome segment in the backcross lines, the more likely several RFLP markers will be identified in the region near the target gene. The relative positions of the RFLP loci are established by segregation analysis, producing a linkage map of the chromosome around the target gene.

This strategy is being utilized to identify the *Ml-o* locus in barley [71]. This locus on barley chromosome 4 does not conform to the gene-for-gene system since recessive alleles determine resistance to all known races of *Erysiphe graminis* f. sp. *hordei*. In an attempt to identify very tightly linked RFLP markers, backcross lines carrying eight different *ml-o* alleles from six different genetic backgrounds were used. These lines had been produced through seven backcrosses of different mutants with one recurrent line. An initial screening procedure eliminated clones encoding repetitive or organellar sequences from a genomic barley DNA library. Among approximately 1,100 genomic clones representing single or low copy DNA sequences, five clones were identified which display RFLPs between DNA from the recurrent line and DNAs from several of the eight backcross lines. Their location on chromosome 4 was corroborated using a barley chromosome 4 addition line in wheat. Genetic distances between four of the

marker loci were found to be 8.6 cM as determined in F_2 individuals of a wide (interspecies) cross between *Hordeum spontaneum* subsp. *spontaneum* and *Hordeum vulgare* subsp. *vulgare*. The genetic distances between the RFLP markers and *Ml-o* were determined in homozygous resistant F_2 individuals from an intravarietal cross between an *Ml-o* line and a mutant carrying allele *ml-o 11*. The results from this cross were consistent with those from the wide cross and a partial chromosomal map including the RFLP marker loci around the *Ml-o* locus could be established. Based on cosegregation of the RFLP markers with the homozygous resistant F_2 individuals *Ml-o* was located to a 2.4 cM RFLP interval. Recently, a new RFLP marker was identified which maps 0.3 cM telomeric of the *Ml-o* locus and serves as a starting point for a chromosome "walk" [Schulze-Lefert, pers. communication].

Identification of resistance genes by chromosome "walking"

Once the region of the genome has been sufficiently narrowed, the marker-flanked DNA can be cloned in a series of overlapping recombinants by the technique of chromosome "walking". In this procedure, the DNA segment from one end of a clone is used to identify adjacent overlapping clones until the entire region of interest has been spanned. The large DNA fragments required by this technique which exceed the capacity of cosmid vectors (35–45 kb) may be cloned using the yeast artificial chromosome (YAC) system [72]. Very large genomic DNA can be partially restriction digested and ligated with the YAC vector arms to yield recombinant DNA fragments of up to 500 kb. Overlapping libraries generated by this method can then be used for chromosome "walking". The final product is the development of a contiguous series of overlapping genomic sequences which is likely to contain a limited number of genes. The final question of which encodes the resistance gene can be answered either by complementation experiments or through comparative DNA sequence analysis of resistance alleles and nonfunctioning mutant alleles.

The vulnerable point of map position-based cloning is the link between genetic and physical distances. As mentioned before, in particular most cereals have tremendous genome sizes. In maize, for example, 0.5 cM correspond to an average of approximately 10^6 bp. In addition, the ratio between genetic and physical distances can vary from locus to locus. Therefore, there is no general answer to the primary question of how close RFLP markers must be linked to a resistance gene to allow a chromosome "walk" in a realistic time.

Transposon Tagging

Transposons, DNA elements which are capable of moving from one location in the genome to another, have been characterized in a variety of organisms including several plant species. Upon transposition, the element may integrate within a gene, thereby inactivating it. If the resultant mutant phenotype can be readily identified, the gene can be isolated using the cloned transposable element as a molecular probe [73, 74].

Best studied among plants are the transposons of maize [75] and *Antirrhinum majus* [76]. This approach is therefore currently being used to isolate resistance genes from maize. Maize transposable elements such as the *Ac/Ds* system, however, have been introduced into and shown to be active in the genome of plants such as tobacco [77], potato [78], tomato [79], carrot and *Arabidopsis thaliana* [80]. This extends their potential as powerful tools for the identification of resistance genes to other species.

The *Rp 1* locus of maize determines resistance to the rust fungus *Puccinia sorghi*. Based on genetic studies and response to different fungal races, 14 dominant resistance factors, $Rp\,1^A$ through $Rp\,1^N$, had been considered an allelic series at this locus [81, 82]. Recently, an analysis of the genetic fine structure of the locus using closely linked RFLP markers revealed, however, that several of these resistance factors are probably separate genes rather than alleles which are clustered within about 0.5 cM of each other [83, 84]. In an attempt to clone the *Rp 1* locus, the *Mutator (Mu)* transposon system was introduced into maize lines carrying the $Rp\,1^F$ gene [85]. *Rp 1* homozygous lines with *Mu* activity (♀) were then crossed to an *rp 1* homozygous line (♂). Among the heterozygous F_1 progeny, 38 out of 35,356 seedlings were susceptible to a mixture of eight distinguishable rust races whereas in a standard background, only 1 of 7,339 seedlings was found to be susceptible. To verify the inactivation of the $Rp\,1^F$ locus, all putative $Rp\,1^F$

mutants were self-crossed or backcrossed to the *rp1* tester. 25 of the F_1 plants yielded only susceptible progeny. When the $Rp1^F$ mutants were tested for susceptibility to the individual rust races, most were susceptible to all races while 5 retained resistance to subsets of races, consistent with a gene-for-gene system. Importantly, the resulting race-specific resistance profiles were different from all known resistance profiles of other *Rp1* alleles/genes. The race-specificity tests of the confirmed $Rp1^F$ mutations detected 4 new resistance profiles suggesting that this locus consists of at least 4 separate genetic determinants.

Several other rust resistance genes, *Rp5*, *Rp6*, and *Rpp9*, the latter determining resistance to *Puccinia polysora*, were mapped close to *Rp1* on the short arm of maize chromosome 10 [83]. The occurrence of resistance gene clusters appears to be more common and has also been described for other plants [33, 54, 86]. This raises the question of whether the chromosomal region evolved by gene duplications [83, 84]. Another observation points in the same direction: the inherent instability of the *Rp1* locus [83, 85]. The simplest explanation of some of the patterns of recombination found in test crosses is the occurrence of unequal crossing-over which generally requires linked duplicated sequences [83, 84]. The instability of the locus makes it difficult to distinguish transposon-induced mutants from mutants based on other changes. This is especially true with *Mu*, since this element does not reliably generate germinal revertants or large sector somatic revertants. Due to the presence of tandem repeats, "chromosome walking" may become very complex. Nevertheless, this strategy should eventually lead to the identification of the entire gene cluster.

The functional approach

Unlike the structural approach, analyses of the morphology, physiology and biochemistry of a particular plant/pathogen interaction are the keys for the functional approach of isolating plant resistance genes. The methodological details must therefore be tailored to the pathosystem of interest. Critical to this approach is the availability of genetically defined cultivars, preferably near-isogenic lines, and clonal pathogen races. Such pathosystems must meet two additional specific requirements to permit use of the functional approach. Firstly, the fungus must be amenable to biochemical investigation. It should therefore be capable of growth *in vitro*, excluding most of the obligate biotrophs. Secondly, phenotypic or biochemical markers for the plant resistance response must be identified in order to screen for fungal compounds involved in the elicitation or suppression of the response.

The first goal of the functional approach, the isolation of the complementary avirulence gene and its product, can alternatively be attained using a structural approach, provided the fungus can be crossed. This is a necessity for obligate biotrophic fungi which resist cultivation *in vitro* and biochemical analyses. Due to the large genome size of the eukaryotic fungal pathogens, map position-based cloning represents the preferable strategy. This approach is being followed with the lettuce downy mildew fungus (*Bremia lactucae*) [87–89] and the barley powdery mildew fungus (*Erysiphe graminis* f. sp. *hordei*) [90]. A prerequisite for the application of this technique to clone fungal avirulence genes is the development of efficient transformation methods, in particular for the obligate biotrophs. Successful transformation of 21 pathogenic fungi has recently been published [46–50]. Should it be possible to develop vector systems with selectable markers of general applicability for fungi and to improve transformation frequencies, complementation experiments should be more feasible.

Transposon tagging may also become a strategy to isolate avirulence genes in pathogenic fungi. Transposable elements in yeast have been well characterized [91]. Recently, a retrotransposon-like element was identified in the tomato pathogen *Cladosporium fulvum* [92] although its activity remains to be demonstrated. Should active transposons be found to be widely distributed among fungi, this may provide an explanation for the high genetic variability of some fungal pathogens as well as an additional tool for the identification of fungal avirulence genes.

Identification of fungal avirulence genes

Various models to explain cultivar-specific resistance have been proposed. Three types of effectors are thought to be determinants of this specificity: elicitors of the plant defense response, suppres-

sors of the defense response and host-specific toxins. Elicitors specify avirulence and are likely to interact directly or indirectly with the complementary resistance gene, thereby triggering the defense response in a genotype-specific manner [93]. Suppressors and host-specific toxins are effectors of virulence. Suppression of the plant defense response could be caused by binding of a suppressor to an elicitor receptor, thereby inhibiting the binding of a cultivar-unspecific elicitor. Consequently, the product of an avirulence gene could be a mutated, non-functioning suppressor while the elicitor receptor or a functionally associated protein could represent the resistance gene product. However, the targets of host-specific toxins must be regarded as the products of "susceptibility genes" which upon mutation could become resistance genes. In the following section, attempts to identify plant resistance genes in different pathosystems representing each of the three types of effector systems are described.

Elicitor-based specificity

The simplest physiological interpretation of the gene-for-gene hypothesis is that pathogen recognition and, as a consequence, plant resistance, is based on the binding of an extracellular pathogen elicitor, the avirulence gene product, to a plant plasma membrane receptor, the resistance gene product. Consequently, a resistance genotype-specific reaction in near-isogenic lines should be triggered by a specific elicitor. *Rhynchosporium* leaf scald of barley appears to conform to this model.

Rhynchosporium secalis is an imperfect fungus which grows beneath the cuticle of barley leaves for long periods of its life cycle [94, 95]. Since the fungus does not establish direct contact with the plant plasma membrane, any effector molecule of plant or pathogen origin must cross the plant cell wall. As a perthotroph, the fungus kills host cells in order to gain access to the plant's nutrient supply. The question therefore is whether toxins or other pathogenicity factors that are involved in this process are exploited by the plant in recognizing the fungus. Such a model, if generally applicable, would explain the frequent dominance of avirulence over virulence, since these factors in principle have a positive role for the fungus in pathogenesis.

R. secalis, race US238.1, is avirulent on barley cultivar "Atlas 46" carrying resistance gene *Rrs1*, but virulent on the near-isogenic cultivar "Atlas" lacking this gene [95–97]. Heterozygous F_1 individuals from a cross between the near-isogenic lines displayed an intermediate phenotype. In the F_2 generation, a phenotypic segregation pattern of 1:2:1 was found, demonstrating that resistance is encoded by a single codominant gene [98].

From culture filtrates of race US238.1, three necrosis-inducing peptides (NIPs) were isolated [99]. These peptides caused necrosis upon injection into leaves of both cultivars. Two of them, NIP1 and NIP3, were found to stimulate the activity of the plant plasma membrane ATPase, again in a cultivar-unspecific manner [100]. NIP2, which had no influence on this membrane enzyme, must have a different mechanism of toxicity. When protein extracts from inoculated resistant and susceptible cultivars were analyzed on Western blots, NIP2 could not be detected while NIP3 and a protein crossreacting with NIP1-antisera were found to be present only in the susceptible cultivar [99]. Since the form of NIP1 detected *in vivo* has a higher molecular mass than the culture filtrate-derived form, it is likely to represent the native form of this compound or a precursor of the smaller form. Importantly, the appearance *in vivo* of both NIP1 and NIP3 correlated with lesion development suggesting their relevance in pathogenesis.

R. secalis does not cause a hypersensitive response in barley leaves. However, upon inoculation of primary leaves with fungal race US238.1, the mRNA encoding a thaumatin-like pathogenesis-related (PR) protein accumulated to a much higher level in the *Rrs1* cultivar than in the near-isogenic cultivar lacking *Rrs1* [98]. The cDNA-derived amino acid sequence of this protein was identical to the N-terminal amino acid sequence of a barley PR protein, Hv-1 [101], induced in an incompatible barley/powdery mildew interaction [98]. The cultivar-specific induction of PRHv-1 mRNA was then used to identify fungal elicitors. One of the necrosis-inducing peptides, NIP1, was found to elicit the transient accumulation of high levels of PRHv-1 mRNA in the *Rrs1* cultivar [100]. In the susceptible cultivar, the mRNA accumulation was much lower and delayed. F_1 plants also displayed an intermediate response at this level (unpublished results). Furthermore, NIP1

was detected in culture filtrates only of this particular fungal race while NIP2 was present in all, and NIP3 in almost all other races analyzed [99]. NIP1 therefore is a candidate for the product of the avirulence gene complementary to resistance gene *Rrs1*. To test this hypothesis, experiments are underway to examine the segregation of the NIP1-induced accumulation of PRHv-1 mRNA and the resistance phenotype in F_2 individuals. In addition, the NIP1 encoding gene is being isolated for complementation experiments. Verification of NIP1 as the avirulence gene product would demonstrate that a secreted fungal toxin is utilized by the plant in the recognition process leading to resistance. In this case, a non-essential fungal pathogenicity gene is defined as an avirulence gene by the presence of resistance gene *Rrs1* in the plant (Fig. 2). Consequently, a NIP1-binding protein in the plasma membrane represents the primary candidate for the product of *Rrs1*.

The only fungal avirulence gene cloned to date appears to fit this model as well. The tomato pathogen *Cladosporium fulvum* also grows extracellularly in the leaves of its host plant [102]. Intercellular washing fluids were collected from tomato leaves inoculated with a virulent fungal race. This race is, however, avirulent on other tomato cultivars carrying resistance gene *Cf9*. From these intercellular washing fluids, a cultivar-specific proteinaceous elicitor of the hypersensitive response was isolated [103]. The elicitor is a peptide of 28 amino acids and causes rapid and extensive necrosis only in tomato cultivars of the *Cf9* genotype. As revealed by cDNA sequencing, this peptide represents the C-terminal portion of a translation product of 63 amino acids [104]. Interestingly, a recessive allele of the fungal *avr9* gene apparently does not exist in fungal races virulent on tomato *Cf9* genotypes. The absence of DNA homologous to particular avirulence genes was also described for bacterial pathogens [105]. The *avr9* gene was transferred into a virulent race of *C. fulvum*. As a result, the isolation of transformants avirulent on *Cf9* tomato cultivars represented the final proof that the *avr9* gene is the avirulence gene in interactions with plants of the *Cf9* genotype (de Wit,

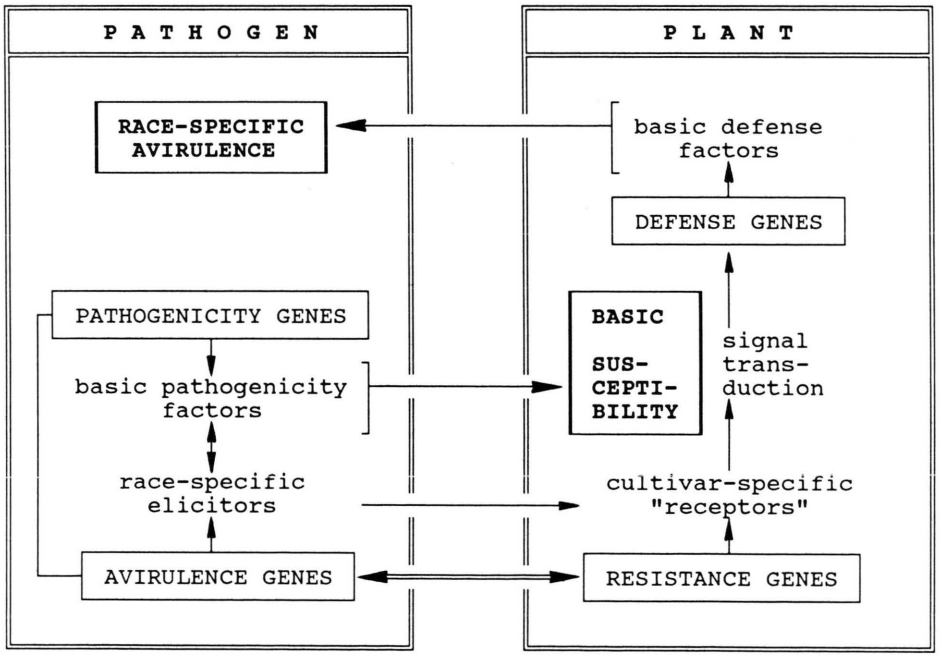

Fig. 2. A physiological interpretation of the gene-for-gene hypothesis. A pathogenicity gene in the pathogen is defined to be an avirulence gene by the presence of the complementary resistance gene in the plant. *Vice versa*, the resistance gene is defined by the avirulence gene. In this model, the avirulence gene encodes a race-specific elicitor, the resistance gene a cultivar-specific elicitor "receptor". Race/cultivar specificity of the interaction is superimposed on basic compatibility.

pers. communication). Since the *avr9* gene is expressed relatively late during pathogenesis on susceptible plants, it may also have a positive, albeit non-essential, function for the fungus in disease development.

The *avr9* gene product in the tomato/*C. fulvum* pathosystem and NIP1 in the barley/*R. secalis* interaction represent elicitors with activities determined by the plant resistance genotype. Alternatively, the function of an avirulence gene product could be to participate in subsequent production of a genotype-specific elicitor. The product of the *avrD* gene of *Pseudomonas syringae* pv. *tomato* conforms to this model [106]. The gene product is a 34 kDa protein which, although not itself an elicitor, appears to catalyze the conversion of a low molecular weight metabolite of several gram-negative bacteria into a specific elicitor of the hypersensitive response in soybean cultivars carrying the *Rpg4* resistance gene [107]. In all three pathosystem described, however, the specificity of the plant/pathogen interaction is based on avirulence gene-associated cultivar-specific elicitors.

Suppressor-based specificity

Pathosystems where a suppressor evokes cultivar-specificity of recognition and defense response [108] are more complicated to analyze. Prior to the isolation of the suppressor, the cultivar-unspecific elicitor must be identified and separated from the suppressor. The wheat stem rust (*Puccinia graminis* f. sp. *tritici*) disease may represent such a system. Fungal race 32 is avirulent on cultivar "Prelude", carrying resistance gene *Sr5*, and virulent on a near-isogenic *sr5* line. A glycoprotein was isolated from fungal germ-tube walls which binds to wheat plasma membranes and which elicits the hypersensitive response in a cultivar-unspecific manner [109, 110]. The fact that membrane binding and elicitor activity could be found with other wheat cultivars as well as with barley suggests that this compound has a role in non-host resistance. Since there are indications of the presence of a suppressor in the intercellular washing fluids from inoculated wheat primary leaves which interferes with elicitor binding [111], experiments are underway to identify this compound. It remains to be shown whether this suppression is resistance genotype-specific or whether the suppressor has a role in establishing basic susceptibility.

Host-specific toxins

The Victoria blight of oats represents a unique system. The causal agent, *Cochliobolus (Helminthosporium) victoriae,* produces a host-specific toxin, victorin [112, 113]. Oat cultivars carrying the dominant *Vb* gene are susceptible to toxin-producing fungal races and sensitive to the toxin [114]. The product of the *Vb* gene appears to be the toxin target. Recently it could be demonstrated that victorin covalently binds to a 100 kDa protein in leaf slices only from a susceptible *(Vb/Vb)* oat line and not from a near-isogenic resistant *(vb/vb)* line [115]. Some uncertainty remains since the specificity of binding was lost when plant extracts were used instead of leaf slices. Antisera raised against the binding protein crossreacted however with a protein from both oat lines suggesting an only minor difference in the gene product of both lines [116].

The *Vb* locus appears to be closely linked or identical to the *Pc-2* locus conferring resistance to a different pathogen, *Puccinia coronata* [117]. The same gene appears to specify susceptibility or resistance depending on the attacking pathogen. Therefore, the isolation of the toxin target, the putative product of a susceptibility gene in the oat/*Cochliobolus* interaction, may simultaneously lead to the product of a resistance gene in the oat/*Puccinia* interaction.

An additional finding was that low concentrations of victorin induce the synthesis of avenalumins, the oat phytoalexins, as well as chlorosis only in *Pc-2* lines, but not in *pc-2* lines even at a thousand-fold higher concentration [118]. This is another example of a toxin being simultaneously an elicitor of the hypersensitive response in a host of a particular resistance genotype. Since the avenalumins do not significantly restrict the growth of *C. victoriae in vitro,* resistance to this fungus is unlikely to be dependent on the accumulation of these phytoalexins [118]. However, an interesting question arising from these data is whether victorin mimics a cultivar-specific elicitor from *P. coronata* [118].

Identification of resistance genes

Prior to the identification of the corresponding resistance gene products, avirulence gene products must be characterized with respect to expression

by the pathogen during pathogenesis, intra- or extracellular localization and immediate function. The avirulence gene product itself, its product or host-specific toxins can then be used to identify their respective target molecules.

The receptors of cultivar-specific elicitors may have functions in unstressed plants originally unrelated to defense. Very similar molecules, therefore, may also be present in related plant species. Since different pathogens must overcome similar defense systems in order to adapt to a particular plant species or family, common strategies may have evolved in pathogenesis. As a consequence, related molecules may have acquired similar roles in the recognition of related pathogens. This may provide an explanation for the observation that *avrD* and two other genes from *P. syringae* pv. *tomato* function upon transconjugation into *P. syringae* pv. *glycinea* as avirulence genes in the interaction with soybean [106, 107]. Similarly, the transfer of *avrRpt2,* an avirulence gene in the interaction of *P. syringae* pv. *tomato* with *Arabidopsis thaliana,* into virulent strains of *P. syringae* pv. *glycinea* yielded cultivar-specific resistance on soybean [119]. Another example may be the detection of new avirulence genes in the rice blast fungus, *Magnaporthe grisea,* introduced by crossing from a fungal strain, pathogenic on a different grass species but non-pathogenic on rice [120].

Outlook

It should not be long before the molecular identification of resistance genes from different plant species has been achieved. This will eventually offer the possibility of transforming agronomically valuable, but susceptible cultivars, creating resistant cultivars in much less time in comparison with classical breeding. Resistance genes from wild relatives have been introduced into crop plants by breeding. This means that recognition molecules of a wild species can be integrated effectively into the defense system of nearly related cultivars. The interesting question is now whether this is also possible across species boundaries, for example from barley to wheat. Since single-gene resistance has usually been found to be broken however within a relatively short time with the evolution of new pathogen races, gene transfer is not the primary goal in the isolation of genes conferring cultivar-specific resistance. Characterization of the regulation and immediate function of resistance genes will undoubtedly lead to a much greater understanding of plant defense systems in general. Deeper insight into the mechanisms of plant defense together with improved therapeutic methods will give rise to the development of protective agents such as engineered elicitors ("elicitor design") of the natural plant response (induced resistance). Additionally, engineered fungicides ("fungicide design") may be the product of an understanding of how plants protect themselves against particular pathogens. Both are likely to provide greater protection of agronomically important species such as cereals than methods currently in use and potentially in a manner less deleterious to the environment.

Acknowledgements

All colleagues at the Department of Biochemistry, Max-Planck-Institut für Züchtungsforschung, are greatly acknowledged for their critical discussions on the manuscript, Dr. Wendy Sacks in particular for language editing. Part of the results described are from a project funded by the Bundesministerium für Forschung und Technologie through a grant to the author.

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