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Infection structures of plant pathogenic fungi – potential targets for plant disease control

Infektionsstrukturen pflanzenpathogener Pilze – potentielle Angriffsorte für Pflanzenschutzmaßnahmen

Dedicated to Prof. Dr. Rudolf Heitefuß on the occasion of his 70th birthday.

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Summary

In the last years, the use of modern physiological, biochemical, and molecular techniques has contributed to a better understanding of the biology of plant pathogenic fungi. The initial phases of infection are crucial for the establishment of the fungus within the host plant and its successful propagation. Thus, spore attachment to the plant surface, germination, germ tube elongation, appressorium development and penetration into the plant tissue are the targets for early disease control. Knowledge about the molecular details of the infection process bears potential to develop specific fungicides without side-effects to other organisms.

In this article, we give a brief overview of the history of fungicide development. Further, we present some examples of modern fungicides with target sites in early infection structures, and discuss their advantages and disadvantages. Up to now, chemical leads of fungicides have been isolated by random screening. In the last chapter, we describe novel possibilities to develop compounds that are directed against specific molecular targets of plant pathogenic fungi.

Key words: anilinopyrimidine; appressorium; fungicide; spore germination; H^+-ATPase; haustorium; strobilurin

Zusammenfassung

Die Anwendung moderner physiologischer, biochemischer und molekularer Techniken hat in den vergangenen Jahren zu einem vertieften Verständnis der Biologie pflanzenpathogener Pilze beigetragen. Insbesondere die Initialphasen der Infektion, in denen der Pilz sich in der Wirtspflanze etabliert, sind entscheidend für seine erfolgreiche Ausbreitung. Daher sind gerade diese Phasen – Anheften an die Blatt- bzw. Wurzeloberfläche, Sporenkeimung, Keimschlauchwachstum, Appressoriumbildung und Penetration in das Blatt- bzw. Wurzelgewebe – die Angriffspunkte für eine frühzeitige Bekämpfung. Detaillierte Kenntnisse über die molekularen Zusammenhänge des Infektionsverlaufes eröffnen die Aussicht auf die Entwicklung spezifischer Fungizide, die keine Nebenwirkungen auf andere Organismen haben.

In diesem Artikel geben wir einen kurzen Überblick über die Geschichte der Fungizidentwicklung. Wir zeigen dann einige Beispiele moderner Fungizide auf, deren Angriffsorte in den frühen Entwicklungsphasen der pilzlichen Infektionsstrukturen liegen und diskutieren ihre Vor- und Nachteile. Während die Leitsubstanzen dieser Fungizide durch Screeningverfahren isoliert worden sind, zeigen wir in einem letzten Kapitel neue Möglichkeiten auf, gezielt Wirkstoffe für spezifische molekulare Angriffsorte in pflanzenpathogenen Pilzen zu entwickeln.

Stichwörter: Anilinopyrimidin; Appressorium; Fungizid; Sporenkeimung; H^+-ATPase; Haustorium; Strobilurin

1 Introduction

Plant pathogenic micro-organisms, in particular fungi, are responsible for severe yield losses in the main crops world-wide. Amongst the measures to control plant diseases, such as the use of disease resistant cultivars (HARTLEB et al. 1997), crop rotation and improved cultivation practice, the application of chemicals has played a major role since the middle of the last century.

Despite the availability of more than hundred compounds registered as fungicides today, there is a continuous demand for new antifungal chemicals. This is due to an increased awareness of the public for the environmental impact of agrochemicals and the desire for safer products. Furthermore, the development of resistance in the pathogens has made many fungicides less effective with time of their use (KNIGHT et al. 1997). In the last decade, the increased application of cytological and molecular techniques have provided us with a new understanding of the development of plant-pathogen interactions. In this article, we will discuss how the increasing knowledge on fungal infection bears potential for new strategies of plant disease control.

2 Brief overview of antifungal compounds

The first trials to control plant diseases did not care very much about fungal infection strategies. Little was known at that time of hyphae specifically differentiated to pave a pathogen's way into the host tissue. The role of appressoria or penetration hyphae was only speculative. Fungitoxicity, stability to light or adherence to plant surfaces were the factors that counted. Early control agents such as urine (TILLET 1755), sulfur (FORSYTH 1803) or copper (MILLARDET 1886) had toxic effects on many organisms including the plants they were supposed to protect. For instance, copper may injure the fruit surface or it may accumulate in the soil and affect numerous organisms. Early in this century, dramatic improvements were obtained after the introduction of metallorganic compounds such as chlorophenol mercury (RIEHM 1913). Between 1930 and 1960, a great variety of organic compounds was discovered such as derivatives of thiocarbamates and phthalimides. These "broad spectrum" fungicides were mainly used for external protection with little systemic effects.

During the last thirty years, effective fungicides with systemic action and curative protection reached the market. These compounds are taken up by the plant and transported in the apoplast or symplast to the young shoots or even to the roots. They do not interfere substantially with the plant metabolism but influence basic activities of the fungus. They have specific targets as such (1) nucleic acid metabolism and nuclear division, (2) ergosterol biosynthesis, (3) cell wall synthesis, (4) respiration, (5) enzyme secretion involved in virulence or (6) the elicitation of natural defense responses of plants (KÖLLER 1992; LYR and BRAUN 1995). A typical example is the benzimidazole derivative "Benomyl" or "Benlate", that has been introduced in 1967. This fungicide is metabolized to methyl-2-benzimidazolcarbamate within the plant. It is taken up by the fungus and binds to β-tubulin subunits of microtubules. Thus, it interferes with microtubule assembly and inhibits spindle formation, thereby blocking meiosis and mitosis of the fungus. However, a single mutation in the gene encoding β-tubulin renders the target organism resistant to the fungicide (DAVIDSE 1986). Thus, we had to learn that a drug acting on a specific target has the inherent disadvantage of provoking the appearence of resistant fungal populations sooner or later.

3 Modern fungicides against fungal infection structures

The various stages of fungal infection offer a number of specific target sites for plant pathogens. Infection structures are hyphal differentiations of pathogenic fungi for the penetration and colonization of their host organisms. We distinguish several steps of the plant infection process: attachment to and recognition of the plant surface; germination of propagules and penetration of the plant surface; and colonization of

the plant tissue (HARDHAM 1992; MENDGEN et al. 1996). Particularly in the early stages of infection, proper function of infection structures is the main determinant of success or failure of the pathogen.

Because of their unique properties that are not shared by nonpathogenic fungi, it is likely that chemicals that block infection structure function exert a particular degree of specificity. In the following, the phases of infection structure development and some examples of interfering fungicides are described.

3.1 Adhesion

For phytopathogenic fungi, adhesion on the plant surface is essential as a first step for establishing a successful infection. This process mostly requires the secretion of extracellular mucilages – often mixtures of glycoproteins and/or enzymes (NICHOLSON 1996). Thus, the contact usually occurs by hydrophobic interactions of surface molecules. In spite of the employment of foliar fungicides, an effective control of phytopathogenic fungi before attachment seems questionable.

3.2 Germination of germ tube elongation

After attaching to the plant surface, the developmental program of a pathogenic fungus starts with the germination of spores followed by growth of the hyphae on the plant surface. During this time, the fungal structures provide an oprimal target for the classic foliar protective fungicides such as dithiocarbamates and phthalimides. However, these unspecific broad spectrum compounds show side-effects against plants, arthropods and mammals.

A new class of antifungal compounds with high specificity is that of the strobilurins. These broad spectrum fungicides are inhibitors of the respiratory chain in fungi at the highly conserved cytochrome bc1-complex probably by producing a conformational distortion (BECKER et al. 1981). Their low toxicity towards planrs has been explained by different penetration and degradation, rather than by differences in the mitochondrial target sites (KNIGHT et al. 1997). Despite its specificity, the fact that the gene encoding its molecular target is located on mitochondrial DNA delays the speed of resistance development (ZHENG and KÖLLER 1997). In the case of the apple scab fungus *Venturia inaequalis,* the strobilurin kresoxim-methyl inhibits germination of conidia (GOLD er al. 1996). A comparison of *V. inaequalis* populations from orchards that had been treated with kresoxim-methyl over 6 years with populations from orchards that had never been treated with fungicides shows no significant differences in sensitivity to this fungicide (KUNZ et al. 1998).

Another new group of compounds inhibiring germ tube elongation are the anilinopyrimidines – foliar fungicides with excellent activity against grey mould *(Botrytis cinerea)* and apple scab. They inhibit the secretion of lytic enzymes such as cutinases, cellulases and pectinases in *B. cinerea* cultures (MILLING and RICHARDSON 1995). These extracellular enzymes are assumed to play an important role in penetration of plant surfaces and mazeration of cell walls (WALTON 1994; HAHN et al. 1997). Later, it was found that the anilinopyrimidines interfere with the biosynthesis of methionine (MASNER et al. 1994; FRITZ et al. 1997). Biochemical studies with different fungal species showed that the toxicity of various anilinopyrimidines was reversed by addition of a mixture of amino acids to synthetic culture media. This fact seems to be important for evaluation the resistance development, since sensitivity assays with fungal cultures in complex media would result in false estimations.

By using microscopical *in vivo* techniques with inoculated leaves (SIEBELS and MENDGEN 1994) to evaluate the sensitivity of *V. inaequalis* against the anilinopyrimidines pyrimethanil and cydrodinil, KUNZ et al. (1998) analyzed apple orchards which had been treated 43 times over 4 years in comparison with orchards that had never been treated. The results indicated that no resistance has developed. Both, the example of strobilurins and that of anilinopyrimidines show that the knowledge about the fungal infection process and the suitable conditions can help to learn more about the fungicide site of action and to monitor resistance development.

3.3 Appressorium formation

Appressoria are specialized structures at the tips of germ tubes that are firmly attached to the plant surface. Several fungi have developed these structures to penetrate the plant cuticle and/or the cell wall (HOWARD

and VALENT 1996). From the base of the appressorium, a penetration hypha grows out and pushes its way into the tissue by means of pressure and, possibly, the release of cutin- and cell wall-degrading enzymes. In some cases, for example the rice blast pathogen *Pyricularia oryzae* and *Colletotrichum* spp., melanization of the appressorial cell wall is essential for penetration (DEAN 1997). It is believed that the melanin layer of the cell wall decreases its permeability. By this means, the fungus is able to build up an internal turgor pressure by producing large amounts of glycerol (DE JONG et al. 1997). This pressure pushes the penetration hypha through the plant cuticle into the epidermal cell (HOWARD and VALENT 1996).

Several fungicides such as tricyclazole, pyroquilon and the new compound carpropamid interfere with different steps of melanin biosynthesis (KUBO and FURUSAWA 1991; TSUJI et al. 1997). In the presence of these drugs, *P. oryzae* as well as *Colletotrichum lagenarium* and *C. lindemuthianum* form nonmelanized appressoria and are unable to penetrate intact plant surfaces and to establish an infection. The advantage of the melanin biosynthesis inhibitors is that they are nonfungitoxic. Neither conidial germination nor appressorium formation are affected. The specific mechanism of antipenetrant action makes these compounds effective disease control chemicals.

4 Infection structure development: A source for targets of novel drugs and plant defense

In the last decade, our knowledge on the development and function of fungal infection structures has increased enormously. This was mainly due to the application of molecular cloning techniques, combined with the development of DNA transformation systems for phytopathogenic fungi. For several species that are amenable to transformation-based insertion mutagenesis as well as to classical genetics, such as *P. oryzae, Cochliobolus* spp. (several grass diseases) and *Ustilago maydis* (maize smut), mechanisms controlling essential steps in the infection process have been elucidated (DEAN 1997). The crucial experiments in these studies have been the targeted disruption of single genes, followed by phenotypic analysis of the resulting mutants. Genes that are not required for vegetative growth but essential for induction of disease, are commonly called *pathogenicity* genes (or *virulence* genes, if needed only in a quantitative manner; SCHÄFER 1994). A subset of these genes has been shown to be involved in infection structure formation (DEAN 1997). In the following, examples for potential targets of disease control from different stages of fungal infection will be presented.

4.1 Signalling events of appressorium formation

The molecular signal cascade leading to appressorium formation is beginning to be discovered, with cAMP-dependent protein kinases and MAP kinases as essential components (CHOI and DEAN 1997). The lower branches to these pathways are highly specific for pathogenic development and do not seem to affect the general growth characteristics of these fungi. They would, therefore, provide an attractive target for novel agrochemical drug targets. In support of this assumption was the discovery that appressorium formation of certain strains of *P. oryzae* could be specifically inhibited by the α-factor peptide hormone of baker's yeast; in barley, the infectivity of the fungus was strongly reduced by spraying the hormone (BECKERMAN et al. 1997). It was found that the observed effects were due to changes in cAMP metabolism; cAMP levels seem to be critical for the induction of divergent pathways that lead either to sexual or to pathogenic development (KAHMANN and BASSE 1997; ADACHI and HAMER 1998).

The example shows that it is possible to design highly specific molecules that interfere with essential steps of fungal development. It should be noted, however, that this example applies only to fungi such as *P. oryzae* and *Colletotrichum* ssp. for which appressorium formation is essential to penetrate the host plants. Recently, it has been shown that *Cochliobolus carbonum* mutants that are strongly impaired in the formation of (nonmelanized) appressoria nevertheless are fully infectious (TURGEON et al. 1998).

4.2 Plasma membrane H^+-ATPase

The plasma membrane H^+-ATPase (PMA) has been suggested as a new antifungal target (MONK and PERLIN 1994). PMA catalyses the energy-dependent export of protons from the cytoplasm into the

extracellular space, thereby creating an electrochemical gradient. This gradient supports essential functions such as ion and pH homoeostasis, and the secondary active uptake of nutrients from the environment. A number of properties make PMA a particularly attractive protein for the design of specific drugs:

PMA is one of the few fungal proteins that are known to be indispensable for growth (SERRANO et al. 1986). Being a major consumer of cellular energy, it is one of the most abundant proteins in the plasma membrane. PMA belongs to the P-type class of ion-transport enzymes, some of which are known as specific targets for important pharmaceutical chemicals, such as digitalis glycosides, that specifically inhibit the Na^+, K^+-ATPase in the cardiac muscle. PMA and other P-type ATPases are among the best-studied membrane proteins with respect to their genetic, kinetic, topological, regulatory and drug-interaction features (MOLLER et al. 1996). By means of their topology, it is possible to design chemicals that do not need to pass the plant membrane, but act on the outer membrane facing parts of the proteins. This would avoid resistance problems arising from cytoplasmic metabolism or export of anti-PMA drugs. In a case study using the sulfhydryl-reacting reagent omeprazole and the PMA of baker's yeast, MONK and coworkers have demonstrated a highly specific interaction between ligand and target that makes it promising to search for therapeutically useful agents with a similar mode of action (SETO-YOUNG et al. 1997).

In our group, a biochemical and molecular analysis of PMA from the broad bean rust fungus, *Uromyces fabae,* has been performed. The enzyme was found to be active during all stages of infection structure development, however, strongly increased PMA activity was observed in haustoria, the structures that are supposed to be essential for biotrophic nutrient (STRUCK et al. 1996). In order to perform a detailed analysis of this enzyme, yeast was used as a heterologous expression system. By using an elegant genetic system developed by the group of M. BOUTRY (DE KERCHOVE D'EXAERDE et al. 1995), a recombinant yeast strain was created that lacked its endogenous PMA but was now dependent for growth on PMA from *U. fabae* (STRUCK et al. 1998). This strain could be used for the search of specific drugs that act on PMA of rust fungi, very similar to the way described above.

4.3 *In planta*-induced fungal proteins

In the last years, a rapidly increasing number of genes has been isolated from phytopathogenic fungi that are induced during growth in their host plants. For a few of them, an essential role for pathogenicity could be confirmed by targeted gene disruption (TALBOT et al. 1993; HWANG et al. 1995), whereas several others turned out to be not essential. Unfortunately, for many of the economically important biotrophic fungi, such as *Phytophthora* spp., the rusts, the powdery mildews and the downy mildews, a mutagenic approach is still very difficult or impossible because of the lack of DNA transformation systems. Nevertheless, as illustrated below, *in planta*-induced proteins bear considerable potential as future targets for novel drugs.

In our group, a large number of genes from the rust fungus *Uromyces fabae* has been isolated that are specifically activated when the fungus is forming haustoria in the cells of its host plant, *Vicia faba* (HAHN and MENDGEN 1997). One gene (PIG2), encoding a protein very similar to amino acid transporters from other fungi, was found to be almost exclusively expressed in haustoria, supporting the essential role of haustoria for rust nutrition (HAHN et al. 1997). Other *in planta*-induced rust genes were found to be probably involved in the uptake of sugars and the biosynthesis of vitamin B1 (HAHN and MENDGEN 1997; J. SOHN, R. VÖGELE and M. HAHN, unpublished data). By using yeast as an expression system, a functional characterization of the encoded proteins is underway. If any of these proteins turn out as being essential for pathogenicity, they can be used for the identification of specific inhibitory drugs.

4.4 Extracellular fungal proteins as targets for the identification of novel plant resistance genes

Plants have the ability to resist the invasion of the majority of potential pathogens by a battery of inducible defense mechanisms, the most effective one being a controlled suicide mechanism called the hypersensitive cell death. Induction of these defense responses is triggered by recognition of specific

molecules of the pathogens by plant receptors. According to the well-established gene-for-gene concept, avirulent pathogens often carry an avirulence gene whose product can be recognized by the product of a corresponding resistance gene from the plant. Breeding for plant resistance has been based for a long time on the introgression of resistance genes from wild into cultivated species. More recently, the cloning of resistance genes from a variety of plants has opened the possibility to use recombinant genetic techniques and transgenic plants to increase the speed of resistance breeding and to achieve more durable resistance (HAMMOND-KOSACK and JONES 1996).

In the tomato pathogen *Cladosporium fulvum*, two proteins (ECP1/ECP2) have been identified that are secreted by the fungus only during its growth through the intercellular space of the host plant. After cloning and mutagenesis of the corresponding genes, it was shown that ECP2 is a major determinant of pathogenicity, because ECP2 mutants showed significantly decreased colonization (LAUGE et al. 1997). Nevertheless, it is doubtful whether this protein is sufficiently important to use it as a target for the development of antifungal drugs. However, screening of a large number of tomato cultivars for cellular responses after infiltration of ECP2 resulted in the identification of lines carrying a corresponding resistance gene, Cf-ECP2 (LAUGE et al. 1998). From these results, it is tempting to speculate that any one of the molecules that are secreted by a pathogenic fungus during the infection process (hydrolytic enzymes, structural cell wall proteins, toxins) represents a candidate for being an elicitor of a defense response. To test this idea, individual delivery of such molecules (either in purified form or by appropriate host-organisms such as yeast) into large collections of plant cultivars and screening for defense responses will certainly be done in the near future. If they are identified, resistance genes against fungal virulence factors are expected to be durable because they recognize components that are essential for the pathogens.

5 Conclusions

In recent years, major technological advancements (high throughput microscale screening, laboratory robotics, combinatorial chemistry) have allowed to dramatically increase the speed of synthesizing and testing new chemicals. Nevertheless, discovery of promising lead substances for agrochemicals is still based on random screening of their activity against fungal diseases, either by infection assays under controlled conditions, or sometimes by growth tests with the pathogen on artificial media. The advances in molecular biology and the deciphering of complete genome sequences from an increasing number of organism have opened the perspective to understand the molecular interactions of drugs and their targets in the pathogens. Due to these developments, drug-discovery is currently moving from a disease-oriented, chemical approach towards a target-oriented, knowledge-based biomolecular approach. While this revolution has already taken place in the pharmaceutical industry, it is now beginning in the agronomic sector as well.

The search for novel fungicides has been extended to an increasing number of targets that appear to be suitable because of their essential roles in fungal metabolism. Among those are the ABC (ATP binding cassette) transporters, a large family of membrane proteins that can mediate the active efflux of drugs out of the cell, thereby conferring multiple drug resistance. While this type of resistance is well-known for mammalian tumour cells, it has been observed also in a number of fungi (DE WAARD 1997). Recently, an essential role of an ABC transporter for pathogenicity of *Pyricularia oryzae* has been observed (J. HAMER, personal communication). This is likely to stimulate further research into these proteins. Other fungal targets of interest are cell wall biosynthesis (KURTZ 1998) and polyamine metabolism (WALTERS and MACKINTOSH 1997).

Nevertheless, the risk of resistance development against such specific chemicals remains a serious problem and should not be underestimated. These fungicides require permanent field evaluation of resistance data for an assessment of the resistance risk, combined with appropriate measures for their prudent use. However, the availability of a variety of differently acting compounds would significantly expand our flexibility in the protection of crop plants against fungal diseases.

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