Justus-Liebig-University Giessen
Institute of Phytopathology and Applied Zoology

Cell polarity in plant defense and fungal pathogenesis in the interaction of barley with powdery mildew fungi

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submitted by

Krystina Opalski
from France

Supervisor : Prof. Dr. Karl-Heinz Kogel
Supervisor : Prof. Dr. Sylvia Schnell
Dean : Prof. Dr. Wolfgang Köhler

Examination Committee

Chairman	Prof. Dr. Ernest-August Nuppenau
1. Referee	Prof. Dr. Karl-Heinz Kogel
2. Referee	Prof. Dr. Sylvia Schnell
Examiner	Prof. Dr. Bernd Honermeier
Examiner	Prof. Dr. Wolfgang Friedt

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La quête scientifique a cela de remarquable qu'elle presse sans cesse l'homme à se dépasser.

Pascal

(What is striking in the scientific quest is that it pushes continuously the man to surpass hiself)

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List of Abbreviations

AF Actin filaments
AGT appressorial germ tube
Avr Avirulence
Bgh Blumeria graminis hodei
Bgt Blumeria graminis tritici
BI-1 Bax inhibitor -1
bIR Biologically induced resistance
BTH Benzo-(1,2,3)-thiadiazole-7-carbothioic acid *S*-methyl ester
CA constitutively active
Ca^{2+} Calcium
CaM Calmodulin
CC coiled-coil
cIR Chemically induced resistance
cv. Cultivar
CWA Cell wall apposition
DCINA 2,6-dichloroisonicotinic acid
dpi days post inoculation
DsRed *Discosoma* ssp. Red
DsRNAi double stranded RNA interference
GTPase guanosine triphosphatases
hpi hours post inoculation
HR Hypersensitive Response
H2O2 Hydrogen peroxide
ISR Induced systemic resistance
JA Jasmonic acid
LRR Leucine-rich repeat
LZ Leucine-zipper
MAPK mitogen-activated protein kinase
Mla mildew locus A, resistance
Mlo mildew locus O, susceptibility
NBS Nucleotide binding site
NO Nitric oxide
O_2^- Superoxide
R Resistance
RAC *ras related C3 botulinumtoxin substrat*
RAR *required for Mla specific resistance*
RAS *rat sarcome onkogene product*
ROP Rho of plants
ROR *required for mlo specific resistance*
ROI Reactive oxygen intermediates
ROS Reactive oxygen species
SA Salicylic acid
SAR Systemic acquired resistance
SNARE SNAP receptor
STK serine/threonine kinase
TIR Toll / interleukin receptor
TM Transmembrane

1 Introduction

Plant diseases are destructive and threaten virtually each crop grown on a commercial scale. They are controlled by plant breeding strategies that have introgressed disease resistance genes into many important crops, and by the costly deployment of antibiotics and fungicides. However, the capacity for the agents of plant disease – viruses, bacteria, fungi and oomycetes – to adapt to new conditions, overcoming disease resistance and becoming resistant to pesticides, is very great. For these reasons, understanding the biology of plant diseases is essential for the development of durable control strategies.

1.1 Plant Pathogen Interaction

Plants must continuously defend themselves against attack from phytopathogenic fungi, oomycetes, bacteria, viruses, nematodes and insects but disease is rare (Agrios, 1997; Schlösser, 1997). A plant-pathogen interaction in which the pathogen is able to colonize a plant and to complete its life cycle is considered a compatible interaction. Successful pathogen invasion and disease ensue if the performed plant defense is ineffective inappropriate, the plant does not detect the pathogen, or the host defense is suppressed by the pathogen. Other interactions are referred to as incompatible since they do not lead to successful infection and disease. There are three major forms of resistance, leading to partial or full incompatibility, nonhost resistance, host resistance and induced resistance.

1.2 Type of plant resistance to pathogens

1.2.1 Nonhost resistance

Nonhost resistance describes the resistance shown by all cultivars of the plant species to all races of a pathogen that causes disease in other plant species. It is the most common form of disease resistance exhibited by plants against the majority of potentially pathogenic microorganisms (Heath, 2000). Nonhost resistance visibly relies on a complex genetic control and implies a variety of divergent defense components whose induction does not depend on known resistance genes. The molecular basis of this type of resistance comprises preformed and inducible defense mechanisms. Preformed mechanisms are if either the plant is unable to support the niche requirements of a potential pathogen or the plant possesses sufficient preformed defense systems, such as structural barriers or toxic compounds that limit the

growth and/or development of the pathogen. An incompatible interaction of a pathogen and a nonhost plant often induces several different defense signaling cascades, including generation of active oxygen species, programmed cell death or hypersensitive reaction (HR) in infected cells, and induction of *PR* genes (Mysore and Ryu, 2004).

1.2.2 Host resistance

Once a pathogen has overcome nonhost resistance, it has to face plant genotype-specific type of host resistance. Disease resistance may be controlled by the action of single genes/alleles with a major phenotypic effect (qualitative resistance); through the action of many genes, each of small effect (quantitative resistance); or through a combination of both qualitative and quantitative resistance. In pathogens confronted by significant levels of qualitative resistance, avirulence is similarly controlled by single genes – these are typified by the biotrophic interactions of rusts and mildews, for which the gene-for-gene system is the classic model (Flor, 1971). In "gene-for-gene" interactions between plants and theirs pathogens, resistance requires a dominant or semidominant resistance gene (*R*) in the plant, and a corresponding avirulence (*Avr*) gene in the pathogen.

1.2.3 Induced resistance

Induction of resistance through exposure to a pathogen affords enhanced protection of the plant and is termed 'induced resistance". Depending on the inducing agents, one can differentiate biologically induced resistance (bIR) from chemically induced resistance (cIR). bIR can be induced by both virulent and avirulent or non- pathogenic rhizosphere bacteria. In the case of local acquired resistance (LAR), resistance induction is locally restricted. Systemic acquired resistance (SAR) describes the state of enhanced defensive responsiveness throughout a plant resulting from local infection with a necrotizing pathogen, such as in a HR (van Loon, 1997). In many plants, the induction of SAR is preceded by a systemic increase in salicylic acid (SA) levels, and SA is both necessary and sufficient to induce SAR (Lawton et al., 1995; Dong 2001). A special case is given by induced systemic resistance (ISR). ISR is induced by non-necrotizing mutulastic rhizobacteria or cell-wall derived elicitors from these bacteria. cIR is activated by applying natural SA or synthetic agents like 2,6-dichloroisonicontinic acid (DCINA) and benzo (1,2,3) thiadiazole-7-carbothioic acid-S-methyl ester (BTH) (Kogel et al., 1994; Görlach et al., 1996). Other chemicals capable of inducing SAR include jasmonic acid (JA), ethylene (ET), β-amino acids, unsaturated fatty acids, silicon, oxalate, phosphate and DL-dodecylester HCL (Kessmann et al., 1994)

1.3 Plant defense systems

1.3.1 Preformed defense mechanism

Plants have evolved diverse defense mechanisms to defend themselves against pathogen attack. The cuticle and cell wall of epidermal cells represent physical barriers against penetration by pathogens. The preformed compounds such saponin and other alkaloids, which have antifungal activity represent biochemical barriers. Some can be located on the leaf surface, other are found in the cell wall or intracellularly (Agrios, 1997). Another group of preformed defense mechanism embrace cell wall degrading enzymes e.g. glucanases and chitinases that are stored in vacuoles and released upon cell damage (Agrios, 1997; Schlösser, 1997).

1.3.2 Signal recognition and transduction

Apparently, pathogens are recognized by perception of elicitors through receptors that are either located on plasma membrane or in the cytosol (Ebel and Scheel, 1997; Hammond-Kosack and Jones, 1996). According to the gene-for-gene model, an *Avr* gene is responsible for production of elicitor which binds with a specific receptor, the product of a *R* gene. Binding of the elicitor ligand to its receptor initiates a signal transduction chain, putting into operation the plant multiple defense measures (Dangl and Jones, 2001; Nimchuck et al. 2001; Hammond-Kosack and Parker, 2003). So far, R-gene products are divided into fives classes according to their structural domains. Most plant disease resistances (R) contain a series of leucine-rich repeats (LRRs) and a nucleotide-binding site (NBS). They are termed NBS-LRR proteins. The LRRs of a wide variety of proteins from many organisms serve as protein interaction platform, and as regulatory modules of protein activation (Belkhadir, 2004). Some NBS-LRR proteins possess a putative leucine zipper (LZ) or coiled-coil (CC) sequence, or a Toll-interleukin-resistance (TIR) domain (Hutcheson et al., 1998; Nimchuck et al., 2001). The members of second group are cytoplasmic serine-treonine protein kinases (STK) initiating specific defense mechanisms by phosphorylation proteins. The third group of *R*-gene products possesses a transmembrane (TM) domain in addition to an extracellular LRR motif. The fourth group lacks an NBS and instead has a TM and extracellular LRR. The fifth group has a cytoplasmic STK region in addition to an extracellular LRR and a TM.

Avr gene products/proteins are considered to be virulence factors during the colonization of susceptible host plants by a pathogen, but in resistant host plant cultivars, these proteins act as "specific elicitors" of plant defense responses to betray the presence of the pathogen to the

plant surveillance system (Bonas and Lahaye, 2002; Collmer et al., 2002). Like animals, plants have acquired the ability to recognize conserved surface components of microbial pathogens, called pathogen-associated molecular patterns (PAMPs, Nürnberger et al., 2004). PAMPs, also termed non-specific elicitors of plant defense, are often indispensable for the microbial lifestyle and, upon receptor-mediated perception, inevitably betray the invader to the plant surveillance system.

The earliest reaction of plant cell to elicitors is change in plasma membrane permeability leading to calcium (Ca^{2+}) and proton (H^+) influx and potassium (K^+) and chloride (Cl^-) efflux (Ebel and Scheel, 1997). Transient elevation of cytosolic Ca^{2+} concentration was found to be necessary for elicitor stimulation of the oxidative burst consisting of the accumulation of reactive oxygen intermediates (ROIs), including superoxide (O_2^-) and hydrogen peroxide (H_2O_2) (Jabs el al., 1997; Chandra and Low, 1997). Possible mechanisms of ROIs synthesis include plasma membrane-associated NADPH oxidase, cell wall peroxidases, oxalate oxidases and enzymes of the Mehler reaction (Wojtaszek, 1997; Grant and Loake, 2002; Hückelhoven and Kogel, 2003). Ca^{2+} and small G-proteins of the ROP family have been postulated to enhance superoxide production by NADPH oxidase in plants (Park et al., 2000, Romeis et al., 2000; Sagi and Fluhr, 2001; Ono et al., 2001). ROIs act as direct toxicagents against pathogens, catalyze early reinforcement of physical penetration barriers and are involved in signaling later defense reactions, such as phytoalexin synthesis and defense gene activation, HR and protective reactions in healthy tissue against ROIs damage (Baker and Orlandi, 1995, Levine et al., 1994; Jabs et al., 1997). The oxidative burst is often accompanied by the rapid synthesis of nitric oxide (NO) in the infected tissue (Delledonne et al., 1998). Several mitogen-activated protein kinases (MAP kinases) cascades are also associated with the induction of defense responses (Zhang and Kessig, 2001). MAPK cascades are minimally composed of three kinase modules, MAPKKK, MAPKK and MAPK, which are linked in various way upstream receptors and downstream targets mostly transcription factors (Jonak et al., 2002). Incompatible pathogens frequently provoke the accumulation of both benzoic acid and salicylate (SA), with their highest concentrations forming in the immediate vicinity of the infection site (Raskin, 1992; Ryals et al., 1996). A rapid accumulation of JA was also observed in many plant cells in response to various elicitor treatments (Gundlach et al., 1997).

1.3.3 Hypersensitive reaction

Incompatible interactions are frequently associated with the appearance of necrotic lesions containing dead plant cells at sites of attempted pathogen ingress. (Levine et al., 1994). This HR is defined as the death of attacked cell and/or neighboring cells within a few hours of pathogen contact (Agrios, 1997). SA, ROI and NO accumulation are closely associated with the induction of this process (Shirazu and Schulze-Lefert, 2000). In interaction with obligate biotrophic pathogens that form haustorial associations with host cells, plant cell death would deprive the pathogen of access to further nutrients. On the other hand the role of the HR is not clear in the case of necrophic pathogens, which may profit from this type of reaction (Hammond-Kosack and Jones, 1996).

1.3.4 Papilla

Cell wall appositions (CWAs), also called papillae, seem to represent an important barrier to pathogen penetration. CWAs consist inter alia of a complex of phenolics, callose and proteins reinforced by oxidative cross-linking with ROI (Thordal-Christensen et al., 1997; Hückelhoven and Kogel, 2003; Zeyen et al., 2002). Other constituents of papillae can be lignin, cellulose, pectin, suberin, chitin, lipids and even silicon, but also proteins usually found in cell walls, such as hydroxyproline-rich glycoproteins or peroxidases (Aist, 1976). In walls of epidermal cells that successfully prevent fungal penetration, H_2O_2 strongly accumulates, and non-penetrated CWAs are regularly encircled by cytoplasmic aggregations and vesicle-like structures containing H_2O_2, peroxidase and phenolic material for cell wall toughening (Bushnell and Bergquist, 1975; Hückelhoven et al., 1999).

1.3.5 Cytoskeleton in cellular defense

In the plant- microbe interactions, host cytoskeleton remodeling has been observed during attempts by fungi to penetrate a variety of plants (Gross et al., 1993; Kobayashi et al., 1997a; Kobayashi et al., 1997b, Schmelzer, 2002). The main elements of the cytoskeleton are microtubules and actin filament, also called microfilaments. Evidence for crucial role of the cytoskeleton in cellular defense has been provided by using inhibitors of the dynamic polymerization-depolymerization of microtubules and microfilaments. These suggest a relationship between the changes in cytoskeleton architecture, in particular actin filaments, the cytoplasmic rearrangements and defense of fungal invasion (Gross et al., 1993; Kobayashi et al., 1997a; Kobayashi et al., 1997b). Cytochalasin (an actin polymerization inhibitor) induced fragmentation of actin filaments correlated with enhanced penetration efficiency of several non-host pathogenic fungi that normally fail to invade the plant cells in such interactions;

Defense responses visible at the penetration site, such as cytoplasmic aggregation nuclear movement, occurrence of autofluorescent material, papilla formation, callose deposition, protein and carbohydrate accumulation, and remarkably, HR-cell death were largely abolished by treatment with the actin inhibitor, whereas application of microtubule inhibitors had only minor impact (Kobayashi et al., 1997a, Skalamera and Heath, 1996; Skalamera et al., 1997). The major activities of the cytoskeleton, dynamic disassembly and reassembly and mediation of directed transport, are apparently driving the changes in cytoplasmic organization, resulting in the establishment of a specific cytoplasmic domain at the penetration site, with the major task to locally reinforce the cell wall (Schmelzer, 2002).

1.4.Powdery mildew of barley and wheat

1.4.1 Pathogenesis of powdery mildew fungi and barley/wheat

Barley (Division, *Spermatophyta* / Subdivision, *Angiospermae* / Class, *Liliopsida* / Subclass, *Commelinidae* / Order, *Poales* / Family, *Poaceae* / *Hordeum vulgare* L) and Wheat (Division, *Spermatophyta* / Subdivision, *Angiospermae* / Class, *Liliopsida* / Subclass, *Commelinidae* / Order, *Poales* / Family, *Poaceae* / *Triticum aestivum* L.) are crop plants extensively cultivated in different regions of the world, serving as an important food source. They are used for animal feeding and as carbohydrate/protein livestock food and for making of bread and beer.

Powdery mildew fungi are among the major pathogens causing diseases of cereals in the world (Oerke et al., 1994). The name “powdery mildew” reflects the powdery tarnish on the plant surface and the tricking spores that are the result of the non-sexual reproductive phase of the fungus. Powdery mildew is a common and widespread plant disease that is caused by obligate biotrophic ecto-parasite. Powdery mildew fungi (Kingdom, *Fungi* / Phylum, *Ascomycota* / Class, *Plectomycetes* / Order, *Erysiphales* / Family, *Erysiphaceae*) are on barley *Blumeria graminis* f. sp. *hordei* (*Bgh*) and on wheat *Blumeria graminis* f. sp. *tritici* (*Bgt*).

The *Bgh* and *Bgt* infection cycle starts with landing of a wind-blown haploid conidiospore on leaves. After about 1 h, a primary germ tube emerges at one pole of the oval-shaped spore (Kunoh et al., 1977). This is thought to contribute to recognition of the host surface, and firm physical attachment to the leaf, as well as to gain access to host water (Carver and Bushnell, 1983). During the following hours, a second germ tube emerges on the side of the spore, elongates on the leaf surface, and forms at its extremity a swollen hook-shaped structure, the appressorium. Approximately 12-16 h after spore landing, the fungus attempts to breach the

host cell wall beneath the appressorium by means of a penetration peg. In compatible interaction, about 50% - 70% of germinated spores succeed penetration attempts, depending on environmental conditions and genetic variation (Panstruga and Schulze- Lefert, 2003). In case of successful cell wall penetration, the fungus establishes the haustorium, a specialized infection structure with finger-like protrusions, by about 24 h post inoculation. The haustorium invaginates the host plasma membrane and is though to serve as a "feeding" organ for nutrient uptake (Gil and Gay, 1977). After the successful establishment of the haustorium, growth of aerial hyphae takes place on the epidermal surface, and neighboring cell may be attacked to establish further haustoria, leading ultimately to a fine mesh of white mycelium representing a mildew colony. After 4- 5 d of hyphal growth, conidiophores consist of mother cell producing chains of 5-10 conidia, and subsequently massive amounts of conidia are released and wind-blown to initiate a new infection cycle. This asexual spread is the predominant mode of propagation during the spring and summer months in moderate climates. Sexual reproduction takes place towards the end of the season, generating so-called cleistothecia that are over wintering structures. In the cleistothecium, haploid ascospores are produced after caryogamy and meiosis, which occurs in the so-called ascus. Upon release from the ascus, ascospores behave like conidia and are able to start new rounds of infection.

1.4.2 Molecular mechanism of powdery mildew pathogenesis

The development of *B. graminis* is a complex morphogenesis process. It involves the degradation of the conidial cell wall at the site of germ tube emergence, relocation of existing resources or de novo synthesis of metabolites necessary for germ tube and appressorial construction and latter turgor build-up in the distal cell of the AGT. The differentiation of the infection structure follows a tightly defined progression of events, which demand the coordinated perception of multiple external signals and their integration via complex signal transduction cascades (Green et al., 2002). Plant-derived signals for appressorium differentiation in *Bgh* include hydrophobicity and barley cutin breakdown products (Carver et al., 1996; Francis et al., 1996). Cyclic AMP (cAMP) and protein kinase A (PKA) play a role in *B graminis* conidial differentiation (Hall et al., 1999; Hall and Gurr, 2000; Kinane et al., 2000). Furthermore, it was suggested that the mitogen-activated protein kinase (MAPK) pathway also transduces the stimulatory signals and mediates AGT elongation and appressorial formation. Upstream effectors of the cAMP pathway activated MAP kinase suggesting an interaction between the two signal transduction pathways at the level of the heterotrimeric G-proteins (Kinane and Oliver, 2003). Cell wall penetration by *Bgh* is thought

to result from combined cellulase enzyme activity and moderate turgor pressure of 2- 4 Mpa. The contribution of cell wall degrading enzymes was inferred from the presence of fungal cellobiohydrolyase (cellulase) at the appressorial germ tub tip (Pryce-Jones et al., 1999).
The study of transduction cascade in pathogenic fungi is great importance. It allows insight into the role of signaling during morphogenesis and pathogenicity and may yield useful target sites for the design of specific fungicides.

1.4.3 Fungal components of polar growth

Blumeria graminis are filamentous fungi. Localized polar extension of the wall, resulting in a tubular cell shape, is characteristic for filamentous fungi. In general, polarized fungal growth occurs via restriction of the delivery of new membrane and cell wall components to hyphal tips (Wendland, 2001; Momany, 2002). The actin cytoskeleton and microtubules play an important role in this targeted delivery of vesicles to sites of growth (Heath and Steinberg, 1999). In variety of filamentous fungi disruption of actin filaments with cytochalasins interrupts these functions and results in changes in actin distribution, formation of cell wall deposits at sites not destined to expand, reduction in enzyme secretion, growth rate reduction, and ultimately and altered hyphal morphology (Grove and Sweigard, 1980, El Mougith et al., 1984; Torralba et al., 1998a). The actin cytoskeleton is thus required for cell polarity, but also for the establishment and maintenance of hyphal polarity in filamentous fungi (Heath, 1994; Torralba et al., 1998a). As well, the so-called Spitzenkörper is involved in apical growth and behaves as a vesicle supply center directing the traffic of vesicles to the plasma membrane (Bartnicki-Garcia et al., 1995; Riquelme et al., 2002). Proteins, which regulate various aspects of cell growth through the reorganization of the actin cytoskeleton include the Ras and Rho GTPases, including Rho, Cdc42 and Rac (reviewed by Momany, 2002; Harris and Momany, 2004).

1.4.4 Plant susceptibility factors

Susceptibility factors are plant products, which might be required for full susceptibility of a plant during the interaction with a certain pathogen. Barley MLO is a candidate susceptibility factor (Panstruga, 2003). A functional *Mlo* copy is prerequisite for basal compatibility of barley and *Bgh*. The barley *Mlo* encodes the prototype of a plant-specific family of integral plasma membrane proteins that have seven transmembrane helices (Büschges et al., 1997; Devoto, 1999). The wildtype MLO may interfere with CWA-associated processes (Wolter, 1993). Moreover, MLO is predicted to act as a control element of cell death/senescence and as

modulator of mutually inhibitory defense pathways, and is possibly exploited by *Bgh* for "molecular docking" and/or defense suppression (reviewed in Panstruga and Schulze-Lefert, 2002; Panstruga and Schulze-Lefert, 2003). Barley MLO interacts with calmodulin (CaM), a protein that acts as a cytoplasmic calcium (Ca^{2+}) sensor (Kim et al., 2002). A rapid and transient increase of cytosolic Ca^{2+} concentration has been reported as an immediate early host response upon pathogen attack, and is thought to be required to trigger various defense responses (Mithöfer et al., 1999; Blume et al., 2000; Romeis et al, 2001). The decreased complementation efficiency of MLO variants that are defective in CaM binding suggests that increased Ca^{2+} concentration not only might activate defense responses but also promote susceptibility, at least in the barley-*Bgh* interaction.

RACB, a member of the plant RAC/ROP family of RHO-like small monomeric G-proteins may play a role in MLO-mediated susceptibility to *Bgh*. Silencing of *HvRacB* by double-stranded RNA interference (dsRNAi) reduced susceptibility of *Mlo* plants to *Bgh*, whereas overexpression of a constitutively activated $RACB^{G15V}$ (CA RACB) resulted in enhancement of susceptibility (Schultheiss et al., 2002; Schultheiss et al., 2003). Partial susceptibility in the *mlo5 ror1* and super-susceptibility of *Mlo ror1* mutants was not affected neither by dsRNAi of *HvRacB* nor by overexpression of CA RACB. This *Ror1* dependency suggested a link between MLO and RAC/ROP-proteins, because *Ror1* is also required for recessive *mlo*-defect mediated resistance (Schultheiss et al., 2002; 2003; Stein and Somerville, 2002). It has been shown that plant RAC/ROPs like mammalian RAC/RHO, are involved in actin remodeling, localized Ca^{2+} influx, membrane transport during polar growth and production of ROIs (Valster et al., 2000; Yang, 2002). RAC/ROPs have been shown to be crucial for proper root hair and pollen tube tip growth, and constitutive activation of RAC/ROPs involved led to isotropic instead of polarized growth with altered actin cytoskeleton organization (e.g.; Cheung et al., 2003; Fu et al., 2002; Jones et al., 2002; Kost et al., 1999; Li et al., 2003; Molendijk et al., 2001; Yang, 2002).

Another potential factor of susceptibility is BAX inhibitor 1 (BI-1) proteins, which is characterized as suppressor of programmed cell death in mammals and plants. The barley BI-1 is a suppressor of nonspecific background resistance and *mlo*-mediated penetration resistance to *Bgh* when overexpressed in epidermal cells of barley (Hückelhoven et al., 2003). Moreover, upon overexpression in barley, *BI-1* suppresses penetration resistance to *Bgt*, linking barley nonhost resistance with cell death regulation (Eichmann et al., 2004).

1.4.5 Plant resistance factors

Most analyzed defense reactions of barley against an attack by *Bgh* are specified by dominantly or semidominantly inherited resistance genes, *Mlx* (*mildew locus x*) that act race specifically (Jørgensen, 1994). Defense triggering is dependent on the presence of complementary avirulence genes in the fungus, as described by Flor gene-for-gene (Flor, 1971). At least two genetically separable pathways control resistance to *Bgh* (Jørgensen, 1994; Peterhänsel et al. 1997). In the first successful defense can be triggered by a number of race-specific resistance genes (*R* genes; e.g. *Mla*, *Mlg*, *Mlk*) (Jørgensen, 1994) and is almost invariably associated with the activation of rapid host cell death at attempted infection sites (Freialdenhoven et al., 1994). *Mla* allele-mediated resistance involves predominantly the HR of cell containing haustoria, and the induction of numerous defense-related genes (Freialdenhoven et al., 1994; Boyd et al. 1995). One further gene is required for the *Mla12*-mediated expression of the defense reaction in barley against *Bgh*, *Rar1* (required for *Mla12* specified resistance, Freialdenhoven et al., 1994). *Rar1* was identified through mutagenesis and is necessary for the expression of HR. Mutations in the locus lead to the susceptibility of *Mla12*-plants. The semi-dominantly *Mlg* resistance is characterized by both the formation of effective papillae and the HR of the attacked epidermal cells (Gorg et al., 1993). In the second pathway, resistance is exemplified by non-race specific resistance mediated by recessive *mlo* alleles (Jorgensen, 1994). *mlo* allele-mediated resistance operates early when direct fungal penetration of epidermal cells is attempted. Abortion of pathogenesis of *Bgh* in *mlo* plant is tightly linked with localized cell wall remodeling and biosynthesis processes leading to cup-shaped CWAs (Stolzenburg et al. 1984; Wolter et al., 1993). Because the fungus also triggers CWA formation at sites of successful penetration, lack of *Mlo* may contribute to a faster speed of CWA formation and/or compaction (reviewed in Panstruga and Schulze-Lefert, 2002). However, the role of CWAs is not yet clear.

Re-mutagenesis of *Bgh* resistant *mlo* mutants revealed a few partially susceptible plants due to recessive second site mutations in the genes *Ror1* and *Ror2* (required for *mlo* specified resistance, Freialdenhoven et al., 1996). Thus, the *Ror* genes are required for full *mlo*-mediated resistance. *Mlo ror1* and *Mlo ror2* plants are supersusceptible compared to wildtype *Mlo Ror1* and *Mlo Ror2* (Collins et al., 2003). Thus, *Ror* genes do have a role in limiting fungal growth in compatible interactions. The ROR2 protein represents a syntaxin of the plasma membrane. SNARE family potentially involved in exocytosis or vesicle fusion (Collins et al., 2003). Since vesicles were shown to congregate in attacked epidermal cells at site subtending *Bgh* appressoria (Hückelhoven et al., 1999), it is suggested that ROR2 syntaxin may control the CWA formation.

1.4.6 Fungicide for powdery mildew control

Fungicidal products containing dithiocarbamate, quinomethianate and sulphur have long been known as active against powdery mildew. However they are predominantly protective and their persistence and activity are often relatively small, thus control may require frequent applications. In the early 1970s, the situation changed dramatically with the introduction of systemic products that established new standards of control for powdery mildews. The mode of action of these systemic fungicides involved specific inhibition of physiological processes and they were effective at lower concentrations. The first systemic fungicides used against powdery mildew were benomyl, mopholines, tridemorph and dodemorph, and the 2-aminopyrimidines, dimethirimol and ethirimol. Intensive research throughout the agrochemical industry expanded options for powdery mildew control in the 1980s through introduction of several triazoles (sterol demethylation inhibitors, DMIs) and two additional members of the morpholines group, fenpropimorph and fenpropidin. Recent new chemistry has improved standards of disease control still further through the development of strobilurins (Clough and Godfrey, 1998), azoxystrobin, kresoxim methyl, and trifloxystrobin (Margot et al., 1994), a “morpholine” type compound, spiroxamine (Dutzmann et al., 1996), and a phenoxyquinoline, quinoxyfen (Longhurst et al., 1996). One consequence of using of systemic fungicides was to reduce dose rates. This allowed chemistry to seriously challenge plant breeding as a cost effective way to control powdery mildews in cereals.

1.4.7 Resistance to fungicide

The impact of chemical control has been very much tempered by the ease of powdery mildews developing resistance, quickly rendering many systemic fungicides ineffective (Hollomon and Wheeler, 1999; Limpert et al. 1996; Lyr et al., 1999). Indeed, powdery mildew has a number of characteristics, which favor a rapid adaptation rate, such as its relatively short generation time, with sexual recombination throughout the year, and the nature of its spread, as newly adapted pathotypes can be carried relatively quickly by wind over a wide area. For instance, sterol demethylation inhibitors (DMIs) are not used as single compounds against powdery mildew anymore. Likewise, inhibitors of sterol reductase and isomerase, the morpholines, have shown some sensitivity shifts (spiroketalamines are also affected due to cross resistance). Isolates of wheat powdery mildew resistant to both strobilurins and the quinoline fungicides, quinoxyfen were recently detected at a low

frequency in some parts of Europe (www.frac.info). Many different resistance mechanisms are possible:

The modes of action of different fungicides within each of the groups are very similar or the same. Any pathogen population that is resistant to one fungicide within a group will almost certainly be resistant to other members of that same group. The issue of cross-resistance adds a dimension that limits the flexibility for managing resistance. Once resistance develops to one fungicide, others in that group are likely to also become less effective or useless.

In contrast to cross-resistance, pathogen populations have been shown to develop resistance to fungicides in more than one chemical group. The intensive use of at-risk fungicides in different chemical groups without following resistance management principles can result in the development of multiple resistance. Couple multiple resistances across groups with cross-resistance within groups, and the loss of efficacy for a large number of fungicides is possible.

1.5 Objectives of the study

Cell polarity is important for both, plant defense and fungal pathogenesis. Plant cells respond to a variety of internal and external stimuli with rapid and dramatic rearrangements of their cytoplasm. These changes are often mediated by a dynamic cytoskeleton. It has been reported that the plant actin cytoskeleton plays an important role in the early defense responses of plant cells against fungal penetration. Additionally, plant RAC/ROP proteins are involved in actin remodeling during polar growth of root hairs and pollen tubes. However, nothing is known about proteins regulating actin remodeling in plant-microbe interactions. The first aim of this work was to investigate the actin reorganization in epidermal cells of susceptible *Mlo* and resistant *mlo5* barley attacked by *Bgh* and the influence of overexpression and knockdown of a RAC/ROP G-protein on the actin remodeling during the interaction of barley- *Bgh*. The work should provide a survey of the influence of proteins MLO and RACB on the plant actin reorganization under attack from *Bgh*.

The second part of the present work aims at analysing the mode of action of a new systemic fungicide, metrafenone, to control powdery mildew of barley (*Bgh*) and wheat (*Bgt*). A straightforward approach to determine the mode of action was first to evaluate the preventive, curative and eradicative activities of metrafenone comparing in parallel the activities of kresoxim-methyl, spiroxamine and quinoxyfen and second to mimic the effects of

metrafenone on the fungal morphogenesis with specific inhibitors. These experiments provided evidence that metrafenone might affect the fungal cell polarity. For this reason, I then visualized different components of polar growth such as the cytoskeleton, nucleus, cytoplasm, cell wall and vesicles, to control if the fungicide could affect them.

Together, this study is aimed at contributing to the knowledge about the role of cell polarity in both the host and the pathogen during pathogenesis and plant defense. Furthermore, the first insight into the potential mode of action of a novel fungicide might help to support future disease managing strategies.

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The receptor-like MLO protein and the RAC/ROP family G-protein RACB modulate actin reorganization in barley attacked by the biotrophic powdery mildew fungus *Blumeria graminis* f.sp. *hordei*

Krystina S. Opalski, Holger Schultheiss, Karl-Heinz Kogel and Ralph Hückelhoven*

Institute of Phytopathology and Applied Zoology, Justus-Liebig-University Giessen, Heinrich-Buff Ring 26-32, D-35392 Giessen, Germany

Received 14 July 2004; revised 18 October 2004; accepted 22 October 2004.
*For correspondence (fax +49 641 9937499; e-mail ralph.hueckelhoven@agrar.uni-giessen.de).

Summary

Cytoskeleton remodelling is a crucial process in determining the polarity of dividing and growing plant cells, as well as during interactions with the environment. Nothing is currently known about the proteins, which regulate actin remodelling during interactions with invading pathogens. The biotrophic powdery mildew fungus *Blumeria graminis* f.sp. *hordei* (*Bgh*) invades susceptible barley (*Hordeum vulgare* L.) by penetrating epidermal cells, which remain intact during fungal development. In contrast, resistant host plants prevent infection by inhibiting penetration through apoplastic mechanisms, which require focusing defence reactions on the site of attack. We stained actin filaments in a susceptible *Mlo*-genotype and a near-isogenic race-non-specifically resistant barley *mlo5*-mutant genotype using fluorescence-labelled phalloidin after chemical fixation. This revealed that the actin cytoskeleton is differentially reorganized in susceptible and resistant hosts challenged by *Bgh*. Actin filaments were polarized towards the sites of attempted penetration in the resistant host, whereas when susceptible hosts were penetrated, a more subtle reorganization took place around fungal haustoria. Strong actin filament focusing towards sites of fungal attack was closely associated with successful prevention of penetration. Actin focusing was less frequent and seemingly delayed in susceptible wild-type barley expressing the susceptibility factor MLO. Additionally, single cell overexpression of a constitutively activated RAC/ROP G-protein, CA RACB, another potential host susceptibility factor and hypothetical actin cytoskeleton regulator, partly inhibited actin reorganization when under attack from *Bgh*, whereas knockdown of RACB promoted actin focusing. We conclude that RACB and, potentially, MLO are host proteins involved in the modulation of actin reorganization and cell polarity in the interaction of barley with *Bgh*.

Keywords: actin, MLO, penetration resistance, polarity, small RAC/ROP G-protein, susceptibility.

Introduction

The cytoskeleton is a key factor in determining the polarity of dividing and growing plant cells, as well as in interactions with invading pathogens (Mathur and Hülskamp, 2002; Staiger, 2000; Volkmann and Baluška, 1999; Wasteneys and Galway, 2003). However, nothing is known about the proteins regulating actin remodelling in plant–microbe interactions. In the last few years, it has become increasingly clear that the cytoskeleton plays a crucial role in plant–microbe interactions. Host actin remodelling has been observed during attempts by fungi to penetrate a variety of plants. After the first contact between fungal or oomycete pathogens and the host surface, the arrangement of host actin microfilaments (AFs) changes dramatically. This indicates that actin, either directly or indirectly, can act as a sensor of chemical and mechanical signals emerging from pathogens (Gross *et al.*, 1993; Kobayashi and Hakuno, 2003; Kobayashi *et al.*, 1994; McLusky *et al.*, 1999; Schmelzer, 2002; Takemoto *et al.*, 2003; Xu *et al.*, 1998).

Actin microfilament rearrangement has been observed in plant–fungus interactions, including the interaction of barley

(*Hordeum vulgare L.*) with appropriate and inappropriate powdery mildew fungi. In non-host barley coleoptile cells, AFs became radially arranged beneath the contact sites with the inappropriate pea powdery mildew fungus *Erysiphe pisi*. A similar actin rearrangement was observed in barley coleoptile cells inoculated with virulent *Blumeria* (formerly known as *Erysiphe*) *graminis* f. sp. *hordei* (*Bgh*). However, the incidence of rearrangement was much lower when compared with the inappropriate pathogen attack, indicating a potential suppression of AF remodelling by the appropriate fungus (Kobayashi *et al.*, 1992, 1997a). Furthermore, cytochalasin, an inhibitor of actin polymerization, permitted various inappropriate powdery mildew fungi, which normally fail or have a very low penetration efficiency, to penetrate into non-host plants and form haustoria (Kobayashi and Hakuno, 2003; Kobayashi *et al.*, 1997a,b; Yun *et al.*, 2003). Additionally, when cells undergo a hypersensitive cell death reaction (HR), uninfected neighbouring cells show focusing of AFs facing the dead cell (Kobayashi *et al.*, 1994). Cytochalasins were also reported to inhibit HR in response to fungal pathogens including *Bgh* (Furuse *et al.*, 1999; Hazen and Bushnell, 1983; Škalamera and Heath, 1998; Takemoto *et al.*, 1999), which indicates that actin is also involved in non-apoplastic defence mechanisms.

Actin filaments are important routes for intracellular organelle and vesicle transport. Local accumulation of defence-related compounds, such as callose and autofluorescent materials, occurs simultaneously with the radial arrangement of AFs at sites of fungal attack in barley, cowpea and potato (Kobayashi *et al.*, 1997a; Schmelzer, 2002; Škalamera *et al.*, 1997). Likewise, aggregation of autofluorescent material at the penetration site of *Botrytis allii* in onion epidermal cells is associated with polarization of the actin cytoskeleton (McLusky *et al.*, 1999). Cytoplasmic aggregation and expression of defence-related genes was prevented or delayed by pharmacological depolymerization of actin in potato protoplasts or tuber discs, respectively, which had been challenged with a cell wall elicitor from *Phytophthora infestans* (Furuse *et al.*, 1999; Takemoto *et al.*, 1999). Foissner *et al.* (1996) revealed that vesicle dynamics and exocytosis at wound sites was linked to rearrangement of AFs for vesicle delivery to the plasma membrane. Finally, plant nucleus migration close to the fungal penetration site, as well as local accumulation of phenolics and H_2O_2, are dependent on an intact actin cytoskeleton (Gross *et al.*, 1993; Mellersh *et al.*, 2002; Škalamera and Heath, 1998). Hence, the AFs may support the formation of penetration barriers by recruiting defence-related products specifically to the subcellular site of fungal attack.

The barley powdery mildew fungus, *Bgh*, is an obligate biotrophic pathogen that attacks epidermal cells of barley. *Bgh* invades susceptible barley by penetrating living cells, which remain intact during the period of fungal nourishment and reproduction. In contrast, race-non-specifically resistant hosts prevent penetration by apoplastic defence mechanisms that require focusing defence reactions on sites of attempted penetration. After spore landing, the earliest responses of barley include cytoplasm aggregation, the translocation of the nucleus to directly beneath the fungal germ tubes, and cell wall appositions (CWA, synonym: papilla) (Kita *et al.*, 1981; Zeyen *et al.*, 2002). CWAs consist *inter alia* of a complex of phenolics, callose and proteins reinforced by oxidative cross-linking with reactive oxygen intermediates (Hückelhoven and Kogel, 2003; Thordal-Christensen *et al.*, 1997; Zeyen *et al.*, 2002). In walls of epidermal cells that successfully prevent fungal penetration, H_2O_2 accumulates strongly, and non-penetrated CWAs are regularly encircled by cytoplasmic aggregations and vesicle-like structures containing H_2O_2, peroxidase and phenolic material for cell wall toughening (Bushnell and Bergquist, 1975; Hückelhoven *et al.*, 1999). These observations suggest that polar actin reorganization towards sites of attempted fungal penetration is strongly linked to the formation of CWAs and penetration resistance. However, the role of CWAs is not entirely clear, as susceptible plants also build CWAs, and Arabidopsis mutants lacking CWA-associated callose-synthase are surprisingly less susceptible to powdery mildew than the wild type (Jacobs *et al.*, 2003; Nishimura *et al.*, 2003).

The wild-type MLO protein modulates defence responses to *Bgh*, allowing infection of the majority of epidermal cells in susceptible barley (Büschges *et al.*, 1997). Homozygous mutant (*mlo*) alleles of the *Mlo* gene confer a broad-spectrum disease resistance to *Bgh* (Jørgensen, 1992). In *mlo*-barley, such as lines bearing *mlo5* null-alleles, *Bgh* cannot penetrate (Freialdenhoven *et al.*, 1996; Stolzenburg *et al.*, 1984). As phenol cross-linking appears to occur earlier upon infection in *mlo* than in *Mlo*-barley (von Röpenack *et al.*, 1998), accelerated apoplastic defence is expected to be an important component in *mlo*-dependent resistance. Additionally, *mlo5* barley accumulates H_2O_2 at sites of *Bgh* attack more frequently, earlier and apparently to a higher concentration (Hückelhoven *et al.*, 1999, 2000; Piffanelli *et al.*, 2002). Subcellular H_2O_2 accumulation appears to be connected with focal H_2O_2 production in large vesicle-like structures at the site of fungal attack (Collins *et al.*, 2003; Hückelhoven *et al.*, 1999, 2000). MLO represents a potential transmembrane receptor with seven membrane-spanning domains reminiscent of a G-protein-coupled receptor (Devoto *et al.*, 1999). Nevertheless, MLO defence modulation to *Bgh* appears to function independently from heterotrimeric G-proteins, and appears to be regulated by Ca^{2+}-dependent calmodulin interaction (Kim *et al.*, 2002). Although MLO acts as a negative regulator of defence, *Ror1* and *Ror2* genes (required for *mlo*-specified resistance) are required for full expression of both *mlo* resistance and background resistance to *Bgh* (Collins *et al.*, 2003; Freialdenhoven *et al.*, 1996). The ROR2 protein is a syntaxin of the

plasma membrane SNARE family, potentially involved in exocytosis or vesicle fusion (Collins *et al.*, 2003). Additionally, RACB, a member of the plant RAC/ROP family of RHO-like small monomeric G-proteins, might play a role in MLO-mediated susceptibility to *Bgh*. Transient RNA interference by double-stranded RNA (dsRNAi) of barley *HvRacB* reduced susceptibility of *Mlo* wild-type plants to *Bgh*, whereas overexpression of a constitutively activated RACB^{G15V} (CA RACB) resulted in enhanced susceptibility (Schultheiss *et al.*, 2002, 2003). Partial susceptibility in the *mlo5 ror1* and super-susceptibility of *Mlo ror1* mutants was not affected either by dsRNAi of *HvRacB* or by overexpression of CA RACB. This *Ror1* dependency suggests a link between MLO and RAC/ROP-proteins, although CA RACB does not break complete *mlo*-mediated resistance (Schultheiss *et al.*, 2002, 2003; Stein and Somerville, 2002). Plant RAC/ROPs, like mammalian RAC/RHO, are involved in actin remodelling, localized Ca^{2+} influx, membrane transport during polar growth, and production of reactive oxygen intermediates (Valster *et al.*, 2000; Yang, 2002). RAC/ROPs are crucial for proper root hair and pollen tube tip growth, and constitutive activation of RAC/ROPs led to isotropic instead of polarized growth with altered actin cytoskeleton organization (Cheung *et al.*, 2003; Fu *et al.*, 2002; Jones *et al.*, 2002; Kost *et al.*, 1999; Li *et al.*, 1999; Molendijk *et al.*, 2001; Yang, 2002).

In this study, we show that actin cytoskeleton polarization to sites of attempted fungal penetration is intimately connected to penetration resistance, and that actin reorganization is modulated by MLO and RACB in barley attacked by *Bgh*.

Results

Actin filament patterns in barley epidermal cells attacked by Bgh

To study the distribution of the microfilaments in epidermal cells, we stained actin with fluorescence-labelled Alexa Fluor® 488 phalloidin after chemical fixation of barley leaves, and observed actin fluorescence by confocal laser microscopy (Figure 1). In non-attacked control epidermal cells, AFs were arranged transversely or longitudinally along the leaf axis. AFs were arrayed in cortical spirals, helices or parallel to each other (Figure 1a,b). Fine actin meshwork in the cortical cytoplasm and a cage of actin around the nucleus were also observed. Most cells showed prominent cortical actin, and transvacuolar AFs were usually associated with cytoplasmic strands directly connected to the nucleus.

After inoculation with *Bgh*, fungal conidia germinated and formed a primary non-infective germ tube within 1–2 h, which subsequently attached to the leaf surface. The secondary germ tube matured around 12 h after inoculation to form the appressorium. From this, most fungi penetrated the susceptible host between 14 and 24 h after inoculation (data not shown). Epidermal cells built cell wall appositions beneath both the primary germ tube and the appressorium. In resistant Ingrid-*mlo5*, nearly 100% of attacked cells prevented *Bgh* from penetration, and *Bgh* became stuck in CWAs. Attacked cells generally survived fungal penetration attempts. In susceptible Ingrid (*Mlo*), 50–60% of attacking fungi succeeded in penetrating, and established a compatible interaction 24 h after inoculation. About 10% of attacked cells underwent a hypersensitive cell death reaction without apparently being penetrated.

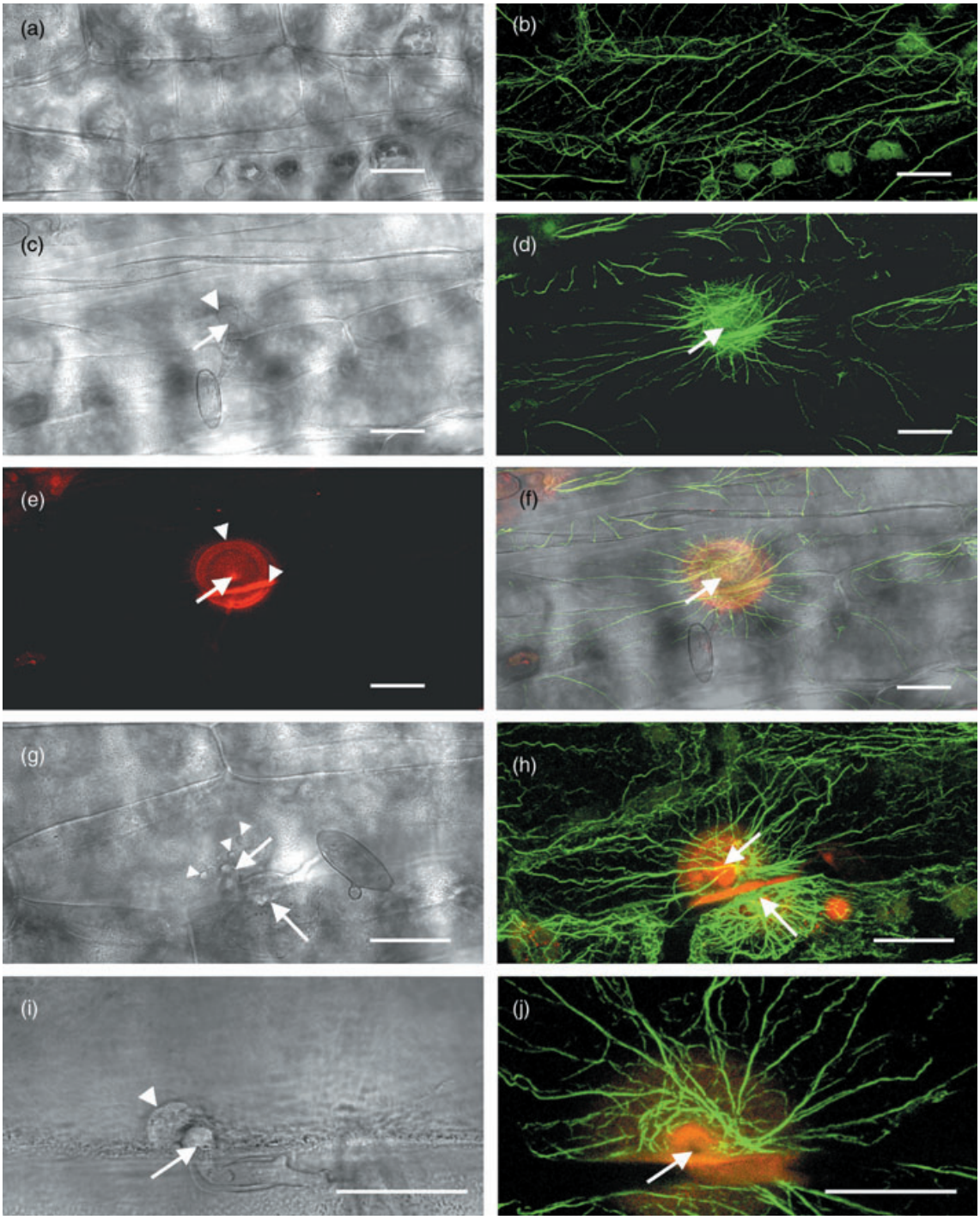
In cells that successfully prevented penetration by *Bgh*, AFs formed a focused pattern. AF focusing occurred beneath both primary and secondary germ tubes at 4 h and 14–36 h after inoculation respectively. At the early pre-penetration stage, only a little AF focusing occurred, although a few transversal AFs started to align towards the site of contact with the appressorium. Several actin bundles connected the nucleus and the site of attempted penetration, while the cortical AFs oriented preferentially transversely or longitudinally to the long cell axis (data not shown). Subsequently, strong actin polarization occurred in most non-penetrated cells. This was characterized by AF focusing and translocation of the nucleus to the site of attempted penetration (Figure 1c–j). Additionally, AFs were often arranged in a dense network of fine filaments surrounding the nucleus (Figure 1i,j). We observed the establishment of apparently new transvacuolar actin filaments oriented towards the site of attempted penetration. In several non-penetrated cells, cortical actin was organized in a concentric meshwork around CWAs. Such concentric meshworks were connected locally to halos of autofluorescent material in the cell wall, expanding over the area of CWA formation (Figure 1c–f,i,j). In non-penetrated cells, either the nucleus, the site of attempted penetration, or both, formed actin organization sites. In some cells, actin staining was diffuse and it was therefore difficult to distinguish insufficient staining from AF disruption. In dead attacked cells, actin patterns were diffuse and blurred by autofluorescence (data not shown).

In contrast, when the fungus succeeded in penetrating the epidermal cell wall, actin polarization was weak. Few AFs focused towards the site of fungal penetration, transvacuolar strands rarely occurred, and the cortical AFs maintained a parallel orientation, transverse or longitudinal to the cell axis (Figure 2a,b). Additionally, in several penetrated cells, AFs closely followed and surrounded the haustorium and the penetrated CWA, reminiscent of cortical actin (Figure 2c,d). Large concentric actin meshworks, as seen around non-penetrated CWAs, never occurred in penetrated cells, although autofluorescent halos were sometimes visible. In several cells, we observed a thick actin filament ring at the tip of an immature haustorium. Occasionally, such actin rings also surrounded single outgrowing haustorial protrusions. Three-dimensional reconstruction and optical

xyz-sectioning allowed visualization of AF rings clearly surrounding the tip of haustoria (Figure 2d,e).

Frequency of actin filament patterns in cells attacked by Bgh

Kobayashi *et al.* (1992, 1997a) found AFs radially arranged at the contact site when *E. pisi*, which is not pathogenic on barley, attempted to penetrate barley coleoptile cells. Although a similar reorganization of AFs was observed in coleoptile cells inoculated with pathogenic *Bgh*, its incidence was much lower than during an attack by the inappropriate pathogen. These results suggest that actin reorganization was involved in penetration resistance of non-host barley. In the present study, we compared the frequency of AF reorganization in near-isogenic-resistant *mlo5*- and susceptible *Mlo*-genotype barley leaf epidermal cells attacked by *Bgh*.



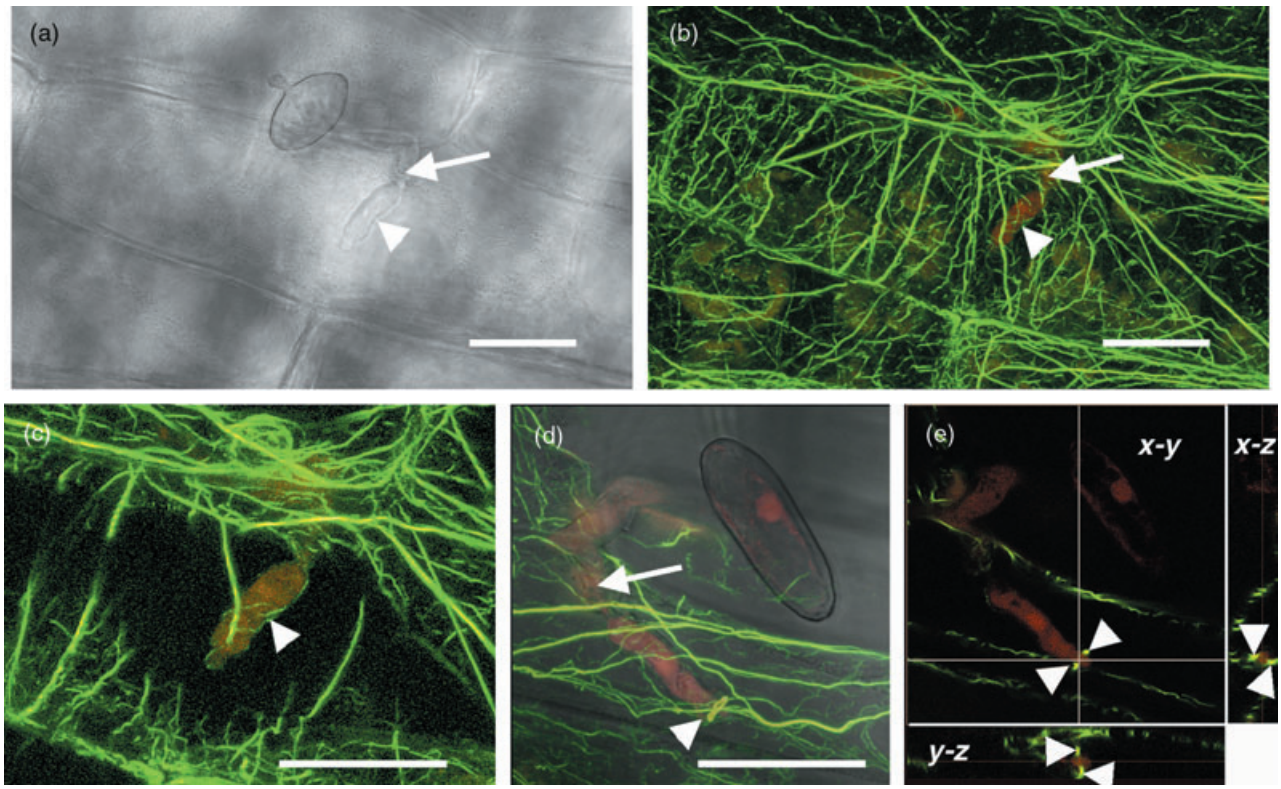


Figure 2. Distribution of actin filaments in susceptible barley (Ingrid) epidermal cells after attack by *Blumeria graminis* f.sp. *hordei*. Actin filaments were stained with Alexa Fluor® 488 phalloidin (green) and imaged by confocal laser microscopy. Microfilaments are shown in green, the fungi by transmission and haustoria by red autofluorescence or transmission. Bars = 20 μm.
(a–c) Same interaction site at 18 hai. (a) Picture shows a penetrated epidermal cell with an immature haustorium (arrowhead) beneath the site of penetration (arrow). (b) Two-colour overlay of two-dimensional maximum projections of 20 optical sections through the entire cell. The overlay of actin fluorescence with autofluorescence of haustorium (arrowhead) shows that only a few actin filaments focus towards the penetration site (arrow), and the cortical actin filaments preferentially arranged transverse to the longitudinal axis. (c) In a stack of 10 middle optical sections, actin filaments closely attached to the haustorium (arrowhead) and the penetrated CWA are visible.
(d, e) Successful haustorium establishment at 24 hai. (d) Overlay projection of two confocal channels (maximum projections) and the transmission channel. The haustorium emerges from the penetration site (arrow) and is led by a ring of host actin filaments (arrowhead). (e) Single optical x-y, x-z and y-z sections through the fungal haustorium. The arrowheads indicate the ring-shaped actin filament bundle around the tip of the haustorium.

We quantitatively assessed changes in AF organization by epifluorescence microscopy and inspection of a large number of cells attacked by *Bgh*. We did not evaluate stomata cells and long epidermal cells above vascular bundles, which show a strong background resistance (Koga *et al.*, 1990).

Twenty-four hours after inoculation, *Bgh* either successfully penetrated host cells, or penetration was efficiently prevented. After the 24-h period, little additional penetration occurred. The frequency of sites with strong actin polarization in non-penetrated cells was 31 and 78% in *Mlo* and *mlo5*

Figure 1. Distribution of actin filaments in resistant barley (Ingrid-*mlo5*) epidermal cells after attack by *Blumeria graminis* f.sp. *hordei*. Actin was stained by Alexa Fluor® 488 phalloidin and imaged by confocal laser microscopy. Microfilaments are shown in green, autofluorescence in red. Fungi and host nuclei were visualized by whole-leaf transmission imaging. Bars = 20 μm.
(a, b) Pictures show a non-attacked cell with actin filaments that form thick bundles oriented transverse to the long axis of the cell and arrayed parallel to each other. (b) Two-dimensional maximum projection of 20 optical sections through the entire cell.
(c–f) Pictures show the same attacked epidermal cell at 18 hai. (c) The site of attack from the fungal appressorium is highlighted with an arrow; the arrowhead indicates the nearby host nucleus. (d) Two-dimensional maximum projection of 10 optical sections through the upper cell periphery. The cortical actin shows radial distribution and a concentric meshwork beneath the site of successful defence in the neighbouring cell. (e) Two-dimensional maximum projection of 10 optical sections through the upper cell periphery. CWA (arrow), the anticlinal and the periclinal cell wall (arrow heads) contain autofluorescent materials. (f) Overlay projection of two confocal channels and the transmission channel (c–e).
(g, h) Figures show an interaction site with double attack from *Bgh* at 24 hai. (g) *Bgh* attacks (arrows) two neighbouring cells. The upper cell reacts with accumulation of large vesicle-like structures (arrowheads) near the CWA. (h) Two-colour overlay of two-dimensional maximum projections of 20 optical sections through the entire cell. The entire actin cytoskeleton of attacked cells apparently focuses towards the sites of attack (arrows).
(i, j) High magnification images of an interaction site with a large CWA at 24 hai. (i) The nucleus (arrowhead) is translocated beneath the site of attempted fungal penetration (arrow). (j) Two-colour overlay of two-dimensional maximum projections of 20 optical sections through the entire cell. Actin fluorescence shows microfilaments that are radially arranged in a concentric actin meshwork around the nucleus and the autofluorescent CWA.

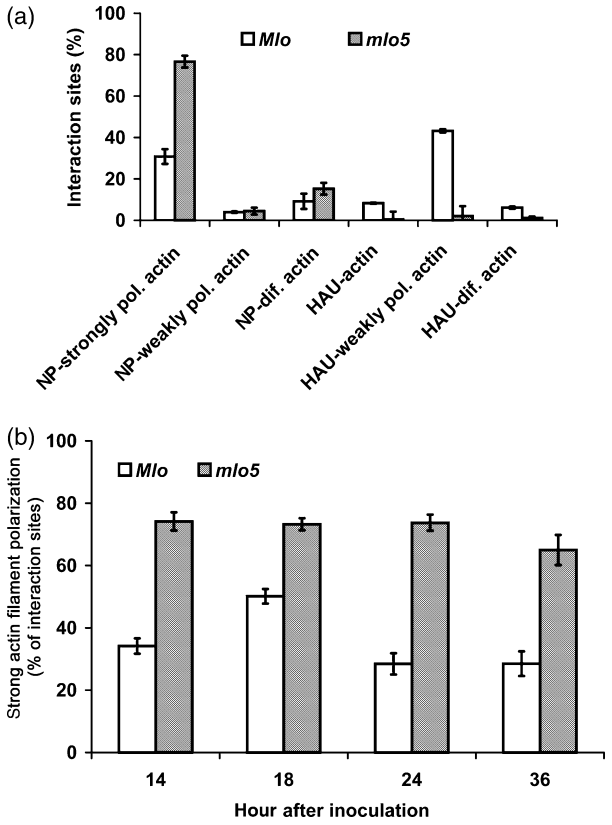


Figure 3. Frequency of actin filament patterns in epidermal cells of susceptible *Mlo* and resistant *mlo5* barleys after inoculation with *Blumeria graminis* f.sp. *hordei*.

(a) Frequency of cells that were either non-penetrated (NP) or allowed haustorium development (HAU), with strongly polarized actin (strongly pol. actin); weakly polarized actin (weakly pol. actin); with diffuse actin (dif-actin); and frequency of the cells in which actin filaments were rearranged closely around haustoria (HAU-actin) at 24 hai. Error bars show standard errors of the mean of three to four independent experiments.

(b) Time course analysis of the frequency of strong actin polarization in epidermal cells of susceptible *Mlo* and resistant *mlo5* barleys at 14, 18, 24 and 36 h after inoculation with *Blumeria graminis* f.sp. *hordei*. Error bars show standard errors of the mean of three to four independent experiments.

plants, respectively, whereas the frequency of non-penetrated cells with weak actin polarization was no more than 2.5% in both genotypes (Figure 3a). Hence, the frequency of successful defence closely correlated with strong actin focusing towards sites of attempted penetration. This was true for both susceptible and resistant barley, although the frequency of successful prevention of fungal invasion and actin polarity was dramatically enhanced in the resistant *mlo5* mutant genotype. In contrast, functional MLO in susceptible barley appeared to counteract strong cell polarity during fungal attack. When the fungus succeeded in forming a haustorium in the *Mlo*-barley cells, the majority of penetrated cells showed weak actin polarization (Figure 3a). Some cells showed subtle actin reorganization around the haustorium and the site of penetration (Figure 2). Rearrangement of AFs around haustoria was difficult to detect by the conventional epifluorescence microscopy used to quantify AF patterns, and therefore might have been underestimated. *Mlo* did not inhibit actin rearrangement in general, but modulated the ability to gain a strongly polar AF organization during attack from *Bgh*.

As the timing of defence reactions and CWA deposition might be crucial in determining the outcome of the interaction, we compared strong actin polarization kinetically in *Mlo* and *mlo5* barley at 14, 18, 24 and 36 h after inoculation (hai) (Figure 3b). During this time course, *Bgh* successfully penetrated about 15–30% of sites by 14 hai and a maximum of about 60% of sites by 36 hai in susceptible *Mlo*-barley. In the resistant *mlo5*-genotype, there was a maximum of 4% penetration at 24 hai. At 14 hai, the frequency of strong actin polarization was 35 and 78% in *Mlo* and *mlo5* barley respectively. In *Mlo* plants, the frequency of strong actin polarization increased to 56% at 18 hai and thereafter decreased to 30 at 24 and 36 hai. In contrast, in *mlo5* plants, the incidence of strong actin polarization remained high at about 80% from 14 to 24 hai, and decreased only slightly at 36 hai. This demonstrates that *mlo5* barley is extremely effective at early polarization of its actin cytoskeleton towards sites of fungal penetration attempts, and at maintaining polarity during the interaction with *Bgh*. Susceptible barley shows an apparently delayed polarization that partially disappears again after establishment of compatibility. Thus functional MLO appears to counteract actin focusing in *Bgh* penetration defence.

To address the question of whether *Mlo*-barley generally fails in AF polarization towards sites of CWA deposition, we quantified AF polarization beneath the non-infective primary germ tube at 4 hai. Strong actin polarization occurred in 49 and 52% of *Mlo* and *mlo5*-barley cells respectively. Hence, during the polarization process in response to this fungal structure, the *Mlo* locus had no clear influence on actin cytoskeleton polarization.

RACB modulates the frequency of actin filament patterns in epidermal cells attacked by Bgh

The barley RAC/ROP protein RACB seems to be involved in the susceptibility mechanism of *Mlo*-barley attacked by *Bgh* (Schultheiss *et al.*, 2002, 2003). In other systems, RAC/ROP proteins modulate cytoskeleton remodelling. To study the potential influence of RACB on AF reorganization in epidermal cells attacked by *Bgh*, we developed a protocol to observe AF patterns in epidermal cells that had been transformed by microprojectile delivery. To identify transformed cells, co-transformation of the red fluorescent protein DsRed and RACB was performed. DsRed moves freely in the cytoplasm and into the nucleus (Dietrich and Maiss, 2002) and can be easily distinguished from the green fluorescence of AFs stained with Alexa Fluor® 488 phalloidin. Non-attacked, transformed cells showed AFs typically arranged as already

seen in non-transformed cells (data not shown). In attacked DsRED-transformed epidermal cells of *Mlo*-barley, as in non-transformed cells, strong actin polarization and nucleus translocation was associated with effective defence (Figure 4a–d), whereas weak polarization was predominant in penetrated cells. Overexpression of the constitutively active form of RACB (CA RACB, Schultheiss *et al.*, 2003) did not influence AF patterns in non-attacked cells (data not shown).

To elucidate the influence of RACB on actin reorganization during the interaction with *Bgh*, we conducted transient knockdown and overexpression of CA RACB in epidermal cells by microprojectile-mediated transformation. Two days after transformation of barley leaf segments, we inoculated with *Bgh*. At 24 h after inoculation, we evaluated the frequency of actin reorganization in transformed cells attacked by *Bgh* by epifluorescence microscopy. Corroborating earlier results of Schultheiss *et al.* (2002, 2003), overexpression of CA RACB relatively enhanced susceptibility of *Mlo*-barley and fungal penetration success by 45%. In contrast, RACB knockdown by dsRNA interference enhanced resistance by 38%. Likewise, in accordance with earlier findings, CA RACB overexpression did not significantly weaken *mlo5*-mediated penetration resistance (data not shown).

As AFs showed a non-polarized pattern in non-attacked cells, we quantitatively evaluated strong AF polarization towards sites of fungal attack at 24 hai as a measure of the ability of transformed cells to reorganize their actin cytoskeleton under attack from *Bgh*. We did not observe clear qualitative changes in AF patterns either after transient knockdown of RACB or after overexpression of CA RACB. In susceptible Ingrid (*Mlo*) control cells transformed with DsRED only, strong actin polarization was observed in as many as 57% of interaction sites, corresponding to a high degree of background resistance in biolistically transformed leaves (Figure 4e). In four independent experiments, *RacB*-dsRNA interference in susceptible Ingrid *Mlo*-barley epidermal cells led to even higher frequencies, with more than 70% of interaction sites showing strong actin polarization (Figure 4e). Overexpression of CA RACB consistently resulted in fewer sites with strong actin polarization. CA RACB significantly reduced strong actin polarization to 39% of sites in *Mlo*-barley. Notably, CA RACB also significantly reduced strong actin polarization in *mlo5* barley from more than 75% to a level seen in susceptible controls, although resistance was not significantly affected (Figure 4e). Together, RACB abundance and activity influenced the ability of attacked cells to remodel their actin cytoskeleton under attack from *Bgh*.

Discussion

We have shown that MLO and RACB influence actin reorganization in barley under attack from the barley powdery mildew fungus. We have also provided evidence for actin reorganization in barley epidermal cells both accessible and inaccessible to *Bgh*. Significantly, actin patterns clearly differed between successfully penetrated and non-penetrated cells, suggesting a role for host actin remodelling in both penetration resistance and haustorium establishment. The process of strong actin filament focusing towards sites of attempted penetration was reduced in a susceptible *Mlo* genotype when compared with resistant *mlo*-mutant genotype. Additionally, the potential host susceptibility factor RACB, in its active form, antagonized AF focusing towards sites of pathogen attack, whereas knockdown of RACB promoted actin focusing.

The actin cytoskeleton plays a crucial role in defence mechanisms during early stages of fungal penetration (Kobayashi *et al.*, 1997a; Schmelzer, 2002). Localized cell wall fortifications and polarized deposition of defence-related compounds at fungal penetration sites are common responses to fungal penetration attempts (Schmelzer, 2002; Zeyen *et al.*, 2002). In our study, we have compared the frequency of AF patterns in cells attacked by *Bgh* in susceptible *Mlo* and resistant *mlo5* plants. By 14–36 hai, the incidence of strong AF focusing towards the site of fungal attack was much higher in *mlo5* than in *Mlo*-barley epidermal cells. We have also shown at the single cell level that effective penetration defence was connected with actin filaments being strongly polarized towards the site of fungal penetration attempts, whereas successful haustorium formation was connected with weak actin polarization in the penetrated cells. The present results confirm previous reports showing that polarization of defence-related materials coincidently occurred with the radial arrangement of the actin cytoskeleton.

In *mlo5* plants, successful penetration defence is associated with earlier and stronger H_2O_2 accumulation in CWAs and in surrounding large vesicle-like structures (Hückelhoven *et al.*, 1999, 2000; Piffanelli *et al.*, 2002). Once reinforced by oxidative cross-linking with H_2O_2, CWAs should be highly resistant to fungal hydrolytic enzymes (Hückelhoven and Kogel, 2003; Thordal-Christensen *et al.*, 1997). In addition, phenol cross-linking in CWAs occurs earlier in *mlo* than in *Mlo* plants (von Röpenack *et al.*, 1998), suggesting that accelerated local defence is an important component in *mlo*-dependent resistance. Foissner *et al.* (1996) reported that rearrangement of AFs was linked to vesicle delivery at the plasma membrane at wound sites, indicating that the actin cytoskeleton might assist wall reinforcement by recruiting cell wall fortification material. In the host barley and the non-host Arabidopsis, a plasma membrane SNARE protein that seems to be involved in vesicle membrane dynamics is required for penetration resistance to *Bgh* (Collins *et al.*, 2003). Thus, in powdery mildew-resistant barley cells the actin cytoskeleton might provide an efficient route for the transport of structural and chemical defence compounds to

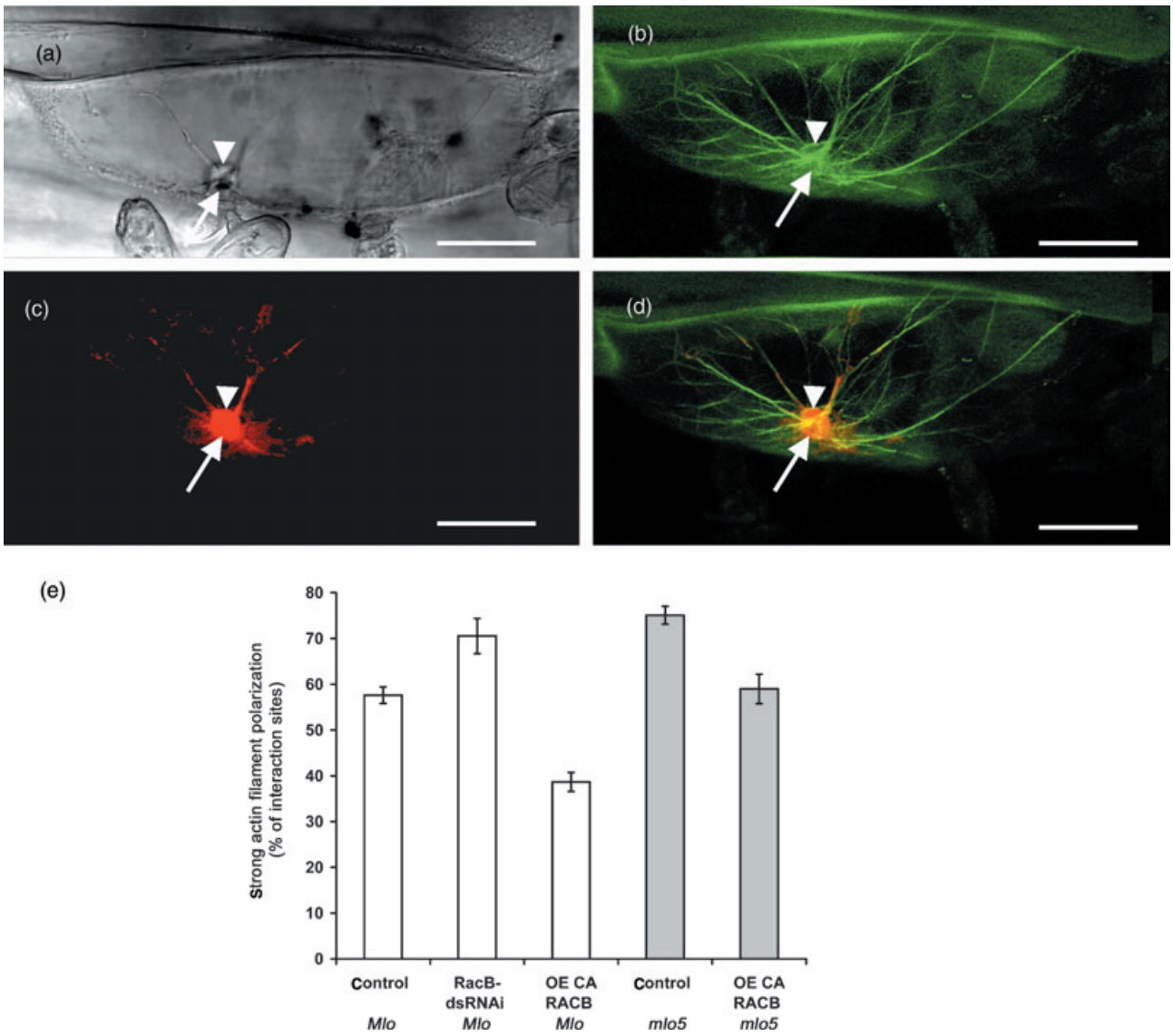


Figure 4. Actin filament patterns in barley RACB transformed epidermal cells after attack by *Blumeria graminis* f.sp. *hordei*.
(a–d) Nonpenetrated DsRED transformed cell showing actin filament focusing towards the site of fungal attack. Bars = 20 μm.
(a) Transmission picture showing the site of fungal attack (arrow) and the host nucleus nearby (arrowhead).
(b) Actin filaments were stained with Alexa Fluor® 488 phalloidin. Actin filaments are arranged in a radial pattern around the site of fungal attack.
(c) DsRed fluorescence in the cytoplasmic strands, cytoplasmic aggregation and nucleus near the site of fungal attack.
(d) A two-channel overlay of actin and DsRed fluorescence shows cytoplasmic strands along the actin filaments focused towards the site of attempted penetration.
(e) Frequency of interaction sites with strong AF polarization towards the sites of attempted penetration in epidermal cells after *RacB*-dsRNA interference or overexpression of CA RACB (OE CA RACB). Columns show the frequency of strong actin polarization. In susceptible Ingrid (*Mlo*), RACB dsRNAi significantly enhanced AF polarization whereas CA RACB lowered the frequency of strong polarization (Student's *t*-test, each $P < 0.05$). In the resistant Ingrid-*mlo5* (I-*mlo5*) CA RACB had a similar effect compared with the effect in susceptible Ingrid (*Mlo*, significantly different from controls; Student's *t*-test, $P < 0.05$). Error bars show standard errors of four repetitions.

the site of attempted penetration. Consequently, strong AF focusing might contribute to a faster formation of penetration barriers. Our results support the hypothesis that actin plays a central role in determining the success or failure of fungal penetration in plant cells by controlling polarization. Wolter *et al.* (1993) suggested that the wild-type MLO may interfere with CWA formation. Accordingly, MLO appears to inhibit or delay actin polarization towards the site of fungal penetration. Additionally, analysis of the time course of strong actin polarization has shown the maintenance of polarity until 36 hai in *mlo5* plants, whereas in *Mlo* plants, polarity declined after fungal penetration. This coincided with transient superoxide production by 18 hai at penetration sites in susceptible *Mlo*-barley (Hückelhoven and Kogel,

1998). In infected *Mlo* cells, the actin cytoskeleton seems to depolarize after the initiation of haustorium development. As this was not observed in resistant barley, depolarization might in fact depend on the influence of a haustorium within a cell rather than on the time after inoculation. Therefore, the fungus might be able to actively counteract host cell polarization, given that MLO is functional.

The *Mlo* locus did not influence actin polarization beneath fungal primary germ tubes at an early interaction stage (4 hai) before an appressorium had been formed. Thus, the *Mlo* wild-type gene does not generally suppress actin polarization to sites of CWA formation. This might be explained by the fact that *Bgh* induces *Mlo* gene expression and MLO protein accumulation between 6 and 12 hai (Piffanelli *et al.*, 2002). Low constitutive *Mlo* expression is possibly not sufficient to suppress polarization. Alternatively, *Bgh* might take over control of the host cytoskeleton by MLO effectors that are released from the appressorium but not from the primary germ tube. Schulze-Lefert and Panstruga (2003) as well as Hückelhoven and Kogel (2003) suggested that *Bgh* might target MLO to suppress host defence. Schulze-Lefert (2004) also suggested that *Bgh* might target MLO to directly interfere with ROR2-dependent polarized secretion of defence compounds. Collectively, the data indicate that functional MLO is required for *Bgh* to modulate polarity in susceptible barley and that such modulation might be part of the fungal defence suppression mechanism.

In successfully penetrated cells, AFs closely followed the haustorium when it invaginated the plasma membrane. During symbiotic, mycorrhizal interactions in tobacco roots, actin filaments followed the fungal branches and enveloped the arbuscule by a dense network (Genre and Bonfante, 1998). In a symbiosis-defective mutant of *Lotus japonicus*, cytoskeleton disorganization and cell death in the presence of a mycorrhizal fungus was observed, suggesting a host factor controlling both at once (Genre and Bonfante, 2002). Parniske (2000) discussed further striking similarities between mycorrhizal and obligate biotrophic parasitic interactions. As actin filaments are linked to the plasma membrane via actin-binding proteins (Baluška *et al.*, 2003), actin remodelling ought to be involved in plasma membrane invagination required for the internalization of a parasitic haustorium. Vorwerk *et al.* (2004) suggested that powdery mildew fungi might have evolved a mechanism that takes advantage of the host papilla response for the establishment of compatibility. The observation that actin cytoskeleton polarization was strong at sites of successful defence, and less strong but detectable when *Bgh* successfully penetrated, seems to support the idea that similar processes are involved in both penetration resistance and compatibility. In particular, the observation of host actin rings at the tip of developing haustoria provokes the view that the host actively takes part in haustorium establishment. As host plasma membrane invagination is a polarized growth process, we named this phenomenon 'inverted tip growth' (Schultheiss *et al.*, 2003). It might even be that a *Bgh* susceptibility factor such as MLO contributes to papilla formation and penetration resistance in other pathosystems. This is supported by the observation that MLO contributes to basal resistance of barley to the rice blast fungus *Magnaporthe grisea* (Jarosch *et al.*, 1999).

The mechanism by which RACB and MLO modulate actin cytoskeleton polarization is not understood. However, based on data in the literature on the role of RAC/ROPs during pollen tube, root hair and mammalian polar cell growth, one may speculate about an RACB-Ca^{2+}-MLO-actin signalling network. Arabidopsis ROP1-6 and barley RACB belong to the same phylogenetic subgroup of type I RAC/ROP proteins (Schultheiss *et al.*, 2003). Collectively, such Arabidopsis RAC/ROPs are involved in establishment and maintenance of polar growth in leaf epidermal cells, root hairs and pollen tubes. The individual members of this RAC/ROP subgroup have partially overlapping but unique functions in the induction of subcellular Ca^{2+} gradients or polar actin patterns, or both. Most constitutively activated type I RAC/ROP mutants induce actin depolarization and isotropic instead of polar growth (Fu *et al.*, 2002; Jones *et al.*, 2002; Kost *et al.*, 1999; Li *et al.*, 1999; Molendijk *et al.*, 2001). Overactivation of RACB-like Arabidopsis proteins seems to inhibit establishment of polarity. Wild-type RAC/ROPs are involved in Ca^{2+} gradient establishment and focal organization of F-actin. There is indirect evidence for Ca^{2+} influx into barley cells attacked by *Bgh* because apoplastic [Ca^{2+}] decreases in susceptible barley attacked by *Bgh* when the fungus attempts to penetrate (Felle *et al.*, 2004). Furthermore, functional MLO depends on Ca^{2+}-mediated calmodulin binding to completely fulfil its role in susceptibility to *Bgh* (Kim *et al.*, 2002). Therefore, RACB might be involved in Ca^{2+} ion flux regulation and establishment of subcellular Ca^{2+} gradients required for both focal actin remodelling and Ca^{2+}-dependent antagonism by MLO function. CA RACB overexpression might induce diffuse instead of focal Ca^{2+} influx, which should interfere with polarization. In such a scenario, RACB would act upstream of MLO, explaining why CA RACB does not break *mlo*-mediated resistance but influences the actin cytoskeleton in *mlo*-barley. Furthermore, RACB might regulate a barley actin depolymerization factor (ADF, synonym: cofilin) required for actin remodelling during apoplastic defence. This appears possible because the tobacco type I RAC/ROP NtRAC1, which is 91% identical and 95% similar to barley RACB, regulates NtADF1 indirectly via its phosphorylation status (Chen *et al.*, 2003), suggesting that constitutive NtRAC1 activity leads to phosphorylation of NtADF1 and overpolymerization of the actin cytoskeleton, which inhibits actin dynamics. Consistent with this, Arabidopsis type I RAC/ROP CA AtRAC1, which is 89% identical and 94% similar to CA RACB, blocked actin depolymerization

during stomatal closure induced by abscisic acid (Lemichez *et al.*, 2001). Our results support this hypothesis because CA RACB-expressing cells were unable to remodel their actin cytoskeleton during attack by *Bgh*. Accordingly, we did not detect less AFs in CA RACB-expressing cells, but an inability to focus AFs towards sites of attempted penetration. In contrast, RACB knockdown might lead to little phosphorylated ADF that should be active in actin remodelling and establishment of polarity, as recently shown in *vivo* for mammalian MTLn3 carcinoma cells (Ghosh *et al.*, 2004).

A direct role of RACB in actin dynamics is supported by the observation that AF polarization could be partially inhibited in *mlo*-barley by CA RACB overexpression (Figure 4), although resistance remained unaffected (data not shown, Schultheiss *et al.*, 2003). This shows that the influence of CA RACB on AF polarization can be uncoupled from the susceptibility-inducing effect, and shows that AF depolarization is not sufficient to break *mlo5*-mediated resistance. Interestingly, infiltration of 3 or 20 μg ml^{-1} of the actin polymerization inhibitor cytochalasin A into *mlo*-barley had a significant but comparatively weak effect on penetration resistance to *Bgh* (data not shown), when compared with the effect on barley coleoptile resistance to *E. pisi* (Kobayashi *et al.*, 1997a). Thus in contrast to depolarization, disruption of the actin cytoskeleton appears to break complete penetration resistance in different experimental designs.

RacB dsRNA interference induces partial *Bgh* resistance (Schultheiss *et al.*, 2002, 2003). In *Agapanthus umbellatus* pollen tubes, antisense oligonucleotides of a *Rop* gene still allowed establishment of a limited Ca^{2+} gradient and FM1-43 dye endocytosis. However, dye uptake was massively slowed down, suggesting a role for RAC/ROPs in membrane dynamics (Camacho and Malhó, 2003). Therefore, RACB might be involved in plasma membrane invagination allowing haustorium establishment, and RACB knockdown cuts such a process down. This idea is supported by the observation that, in animal systems, RAC and RHO proteins are required for macropinocytosis, and bacterial pathogens trigger their uptake into non-phagocytic cells via host RAC and RHO signalling (Knodler *et al.*, 2001; Symons and Rusk, 2003). RACB knockdown might also reduce the Ca^{2+} influx, which should then limit MLO function in susceptibility. This is supported by the observation that AtRop1 antisense RNA reduced pollen tube elongation at low but not at high Ca^{2+} concentrations (Li *et al.*, 1999). ROP knockdown slowed down, but did not abolish, polar growth in pollen tubes while polarity was maintained (Camacho and Malhó, 2003; Li *et al.*, 1999). Similarly, we have shown that RACB knockdown still allows this process, and even enhances it. Possibly, AF polarization is not antagonized by MLO because MLO function is limited when RACB abundance is reduced. Together, MLO and RACB proteins are involved in signalling for host actin cytoskeleton remodelling during defence against an invading fungal pathogen. At the same time, virulent *Bgh* might overactivate RACB, which leads to inhibition of host actin cytoskeleton dynamics and hence polar defence, finally allowing access to epidermal cells.

Experimental procedures

Plant material, pathogen and inoculation

The barley (*H. vulgare*), cv. Ingrid (*Mlo*), and the backcross line Ingrid-*mlo5* were obtained from P. Schulze-Lefert (Max-Plank-Institut for Plant Breeding, Cologne, Germany). Their generation has been described previously by Kølster *et al.* (1986). Plants were grown in a growth chamber at 18°C with 60% relative humidity and a photoperiod of 16 h (240 μmol photons m^{-2} sec^{-1}). Inoculation of the first leaves was performed with 50–100 conidia mm^{-2} from the barley powdery mildew fungus *Blumeria graminis* f sp. *hordei* (*Bgh*), race A6, on the seventh day after germination. The fungus was cultivated on barley cv. Golden Promise under the same conditions.

Transient transformation

A transient transformation protocol, originally developed for wheat to assess gene function in the interaction with powdery mildew, was used to deliver the overexpression constructs and *RacB-ds*RNA into epidermal cells of barley leaf segments as described by Schweizer *et al.* (1999, 2000) and Hückelhoven *et al.* (2003). Barley leaf segments were bombarded with particles coated with pe35SascloptRed (DsRed expression vector, Dietrich and Maiss, 2002) as a transformation marker and either the empty vector pGY1 (Schweizer *et al.*, 1999) or the test-gene construct pRacBV15 (Schultheiss *et al.*, 2003) 48 h before inoculation with *Bgh*. DsRed expression construct was obtained from E. Maiss (University of Hannover, Germany).

To induce RNA interference, we used biolistic delivery of dsRNA into epidermal cells of barley leaf segments as described by Schweizer *et al.* (2000). In parallel control experiments, human thyroid receptor dsRNA was used. Principally, for both overexpression and RNAi, 312 μg of 1.1 μm tungsten particles was coated with dsRNA (2 μg) together with 1 μg pe35SascloptRed for each shot. Double-stranded *RacB* RNA was obtained by annealing of sense and antisense RNA synthesized *in vitro* (Schweizer *et al.*, 2000).

Cytochemistry of actin

Microfilaments were stained as described previously (Kobayashi *et al.*, 1992, 1997a) with slight modifications. Leaf segments 5 × 5 mm in size were fixed in 4% formaldehyde in 25 mM piperazine-*N*, *N*′-bis (2-ethanesulfonic acid) buffer (PIPES, pH 6.8) with 2 mM EGTA, 2 mM $MgCl_2$ and 0.05% Tween 20 (w/v), at room temperature for 1 h. After washing in 25 mM PIPES and in 25 mM phosphate buffer (PB, 4.0 g NaCl, 0.1 g KCl, 0.7 g Na_2HPO_4 $2H_2O$, 0.1 g KH_2PO_4 in 500 ml water, pH 6.8), leaf segments were treated with 0.5% Triton X-100 in 25 mM PB, pH 6.8, at room temperature for 1 h. The specimens were washed with 25 mM PB, pH 6.8, then with 25 mM PB, pH 7.4. Following a rinse cycle, leaf segments were stained with Alexa Fluor® 488 phalloidin (Molecular Probes, Eugene, OR, USA; 0.66 μM in 25 mM PB, pH 7.4) at room temperature for 2–3 h. Vacuum infiltration was repeated three times for

20 sec at 25 mmHg to promote infiltration. Finally, the leaves were rinsed with 25 mM PB, pH 7.4. The specimens were mounted in 25 mM PB, pH 7.4 on glass slides and observed by confocal laser scanning microscopy.

After microprojectile-mediated transformation of the leaf tissues, leaf segments were fixed for 3 h, followed by 6 h phalloidin staining.

Evaluation of fungal development and cytoskeleton arrangements

The different intracellular actin patterns were counted at 4, 14, 18, 24 and 36 hai by fluorescence and bright field light microscopy. For each time, a total of 100 interaction sites were scored on five leaf segments. For statistical analysis, experiments were repeated three to four times. Where differences between *Mlo* and *mlo* genotypes are stressed in the results, genotypes were significantly different with at least $P < 0.05$ (Student's *t*-test).

Actin microfilament patterns were judged as strongly polarized when all thick AF bundles observed by epifluorescence microscopy (excitation, 450–490 nm; emission, 505–530 nm) were directed towards the site of attempted penetration. AF patterns were considered as weakly polarized if only a few AF bundles were focused towards the site of attempted penetration, whilst the majority of MF remained organized longitudinally or transverse to the leaf axis.

Confocal laser scanning microscopy

Confocal fluorescence images were recorded on a multichannel TCS SP2 confocal system (Leica Microsystems, Bensheim, Germany). Alexa Fluor® 488 phalloidin was excited with a 488-nm laser line and detected at 495–518 nm. DsRed was excited with a 543-nm laser line and detected at 580–650 nm. Plant and fungal autofluorescence was excited with a 488-nm laser line and detected at 550–700 nm followed by spectral unmixing from actin fluorescence using the Leica Confocal Software 2.5.1347a.

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Metrafenone – a commercial benzophenone-type fungicide: studies of the mode of action on powdery mildew fungus *Blumeria graminis* ff. spp. on barley and wheat.

Krystina S. Opalski [1)], Ralph Hückelhoven [1)], Stefan Tresch [2)], Karl-Heinz Kogel [1)], Klaus Grossmann [2)], Harald Köhle [2)]

[1)] Institute of Phytopathology and Applied Zoology, Justus-Liebig-University Giessen, Heinrich-Buff Ring 26-32, D-35392 Giessen, Germany

[2)] BASF Agricultural Research Center, APR/FB, Li 470

Postbox 120

67114 Limburgerhof, Germany

Footnotes:

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Corresponding author e-mail: harald.koehle@basf-ag.de, phone: +49 621 60-27635

Abstract

Powdery mildew fungi are among the major pathogens causing diseases of cereals in the world. The mode of action of a novel systemic benzophenone-derivated fungicide, metrafenone has been analyzed on the powdery mildew fungi of barley (*Blumeria graminis* f. sp. *hordei*) and wheat (*Blumeria graminis* f. sp. *tritici*). The preventive treatments significantly reduced germination and blocked development beyond formation of appressoria, which penetrated less often. Moreover, metrafenone turned out to be an efficient eradicative fungicide, which rapidly affected fungal survival at very low concentrations. The fungicide induced swelling, bursting and collapse of hyphal tips resulting in the release of globules of cytoplasm. Bifurcation of hyphal tips, secondary appressoria and hyperbranching were also frequently observed. The cytological analysis showed that metrafenone caused disruption of actin cap and apical vesicle transport as well as weakening of the cell wall at the apex. Finally, metrafenone strongly reduced the sporulation and thus it is an efficient curative fungicide. Reduced sporulation was correlated with malformation of conidiophores, which showed irregular septation, multinucleate cells and delocalization of actin. Microtubules appeared to be only secondarily affected in metrafenone-treated fungi. The results suggest that the mode of action of metrafenone and its potential target are involved in hyphal morphogenesis, polarized hyphal growth, and the establishing and the maintenance of cell polarity. Metrafenone likely disturbs a pathway regulating organization of the actin cytoskeleton.

Introduction

The ascomycete grass powdery mildew fungi, *Blumeria graminis* ff. spp., continue to rank among the most important pathogens on cereals in the world, as reflected by the size of the cereal mildew fungicide market, which is estimated to exceed $300 million per year (Hewitt H., 1999).In temperate regions, it is estimated that, in the absence of fungicides, the powdery mildew fungus would cause approximately 10% yield losses and occasionally as much as 40% (Jørgensen, 1988). Along with cultural measures, main measures of powdery mildew control in the integrated crop protection system are the use less susceptible cultivars and the use of effective fungicides. The main challenge is the remarkable capacity of mildew populations to evolve virulent and fungicide-resistant genotypes that overcome these control measures (De Waard et al., 1993; Limpert et al., 1996, Lyr et al., 1999). Indeed, powdery mildew has a number of characteristics, which favour a rapid adaptation rate, such as its relatively short generation time, with potential sexual recombination throughout the year, and the nature of its air-borne spread. Consequently, finding novel and better active fungicides against powdery mildew fungi represents an important challenge to crop protection research.

The disease powdery mildew is caused by the ectoparasitic obligate biotrophic fungus *Blumeria graminis*: on barley by *Blumeria graminis* f. sp. *hordei* (*Bgh*) and on wheat by *Blumeria graminis* f. sp. *tritici* (*Bgt*). Following contact with the host surfaces, the conidia form a primary germ tube and an appressorial germ tube, approximately 0.5- 2 and 4- 8 h after inoculation, respectively. Papilla formation occurs in the leaf epidermal cell subjacent to the germ tubes. This local response in the outer epidermal cell wall exclude or delay a significant proportion of the attempted penetrations by the fungus (e.g. Carver, 1986). The appressorial germ tube begins to elongate and after 9-12 h differentiates a lobed appressorium. A peg forms under this appressorium, which penetrates the host cell wall and establishes a digitate haustorium within an epidermal cell. Successful establishment of a

haustorium, the only fungal organ that invades the host, is followed by the formation of secondary hyphae from the primary appressorium. An elongating secondary hypha is the starting point for the development of a fungal colony. From the epicuticular hyphae, secondary appressoria are formed and from these, secondary haustoria are established in epidermal cells. Three to four days after the primary infection, conidiophores are formed on the hyphae, sporulation starts and spores can be wind-spread to initiate new infection cycles (Thordal-Christensen et al., 1999).

This report describes the mode of action of a novel systemic fungicide, metrafenone on the powdery mildew fungi of barley (*Bgh*) and wheat (*Bgt*). Metrafenone is the first benzophenone-derivated fungicide and represents an active ingredient that was not used in chemical plant protection before (Koehle et al:, 2004). Metrafenone differs from other commercial fungicides, as its action is different to so far known mechanisms. The research on its mode of action included evaluation of the preventive, curative and eradicative activities of metrafenone, analysis of morphological anomalies of metrafenone-treated *B. graminis*, and visualization of different components of polar growth to see if the fungicide affects these. The results demonstrate that the potential target of metrafenone is involved in hyphal morphogenesis and the establishment and maintenance of cell polarity.

Material and methods

Plant material, pathogen and inoculation

Barley (*Hordeum vulgare* L.), and wheat (T*riticum aestivum* L.) were grown in a growth chamber at 18 °C with 60 % relative humidity and photoperiod of 16h (240 µmol photons/m^2/s). For microscopy, inoculation of first leaves was conducted with 50-100 conidia/mm^2 from *Blumeria graminis* f sp. h*ordei* and *Blumeria graminis* f. sp. *tritici*, at the 7

days after germination. The fungi were cultivated on barley and wheat under the same condition.

Anti-fungal agents

Metrafenone (BAS 560F, 300 g L^{-1} a.i., provided by BASF Aktiengesellschaft, Ludwigshafen, Germany) belongs to the benzophenone fungicide group. It was applied at the concentrations from 0.004 to 250 mg L^{-1}.

To quantify the preventive activity of metrafenone on *B. graminis* in wheat and barley the fungicide was applied on first leaves placed in Petri dishes one day before inoculation. To further determine whether metrafenone had a direct effect on *B. graminis* development, the chemical was applied on inoculated wheat leaves placed in Petri dishes, at 8 hpi and 2 dpi. Subsequently, we assessed mycelium formation and sporulation frequencies at 7 and 14 dpi. Observations were made on 4 pieces of primary leaf segments counting 50 conidia each. These experiments were repeated 4 times. Observations were done by fluorescence microscopy after Uvitex 2B staining.

The effect of metrafenone was compared to that of specific inhibitors. For the inhibition of actin polymerisation, cytochalasin D and A (Molecular Probes, Leiden, The Netherlands), at a concentration of 0.5 mg L^{-1} was used. Benomyl at a concentration of 10 mg L^{-1} was included as anti-microtubular drug. The inhibition of the synthesis of cell-wall chitin was achieved by polyoxin D (Calbiochem, Darmstadt, Germany) at a concentration of 3 mg L^{-1}. Chemicals were prepared with distilled water and applied by spraying until run off with an atomizer on wheat or barley seedlings.

Actin Polymerization Assays

Actin polymerisation assays were carried out using a kit from Cytoskeleton Inc. (Cat. No.: BK003, Labtechnologies, Offenburg, Germany). Pyrene-conjugated actin was polymerized at RT in the presence of ATP and either the compound in question or DMSO. The increase in polymerisation was measured by detecting the increase in fluorescent signal emitted from the pyrene conjugated F-actin using a Fluostar Galaxy (BMG Labtechnologies, Offenburg, Germany) platereader.

Cytological techniques

Uvitex 2B (Ciba Geigy, Basel, Switzerland) was used to stain the β-linked cell wall polymers (Wachsmuth, 1988). Uvitex 2B solved in ethyl alcohol was sprayed directly on the leaves prior to fluorescence microscopy and confocal laser scanning microscopy (CLSM, excitation 365 nm and emission 420- 460 nm).

Staining of the fungal cytoplasm was performed with acridine orange (AO) (Sigma, Steinheim, Germany). AO was prepared as 0.7 % (w/v) solution in sodium acetate buffer (pH 4.0). A 100 mL volume of AO solution contained 700 mg AO in 100 mL acetate buffer (pH 4.0). Acetate buffer contained 82 mL acetic acid (0.2 mol L^{-1}) and 18 mL sodium acetate (0.2 mol L^{-1}). Leaf segments were submerged for 10 min in AO dye solution, and then rinsed with the acetate buffer. The probes were examined immediately by fluorescence microscopy (excitation 490 nm and emission 520- 550 nm).

In order to determine the metrafenone effect on the fungal survival, phloxine B (ROTH, Karlsruhe, Germany) was applied to observe its dead mycelial cell staining property. A 0.1% (w/v) aqueous solution in distilled water was prepared and the samples were plunged in this solution for 20 min followed by rinsing with water. The evaluation was done by bright field microscopy.

Fungal cell walls were stained with congo red (Merck, Darmstadt, Germany), which typically stains crystallized cellulose fibers. The leaf segments were immersed in a solution containing 10μg mL^{-1} of congo red in distilled water, for 20 min. After being rinsed with water, the specimens were directly examined by CLSM (excitation 585 nm and emission 600- 615 nm).

To visualize cytoplasmatic vesicles, we used FM4-64 (Molecular Probes, Leiden, The Netherlands). Leaf segments were placed in 20 mM HEPES buffer (pH 7.2) containing 10 μM of FM4-64, for 30 min. The samples were washed with HEPES and directly observed by CLSM (excitation 414 nm and emission 630- 750 nm).

Staining of nuclei was done with Hoechst 33342 (Sigma, Steinheim, Germany). The leaf segments were immersed in solution containing 1 μg mL^{-1} Hoechst in 25 mM PB, for 10 min. After being washed in PB, nuclei fluorescence was observed by CLSM (excitation 346 nm and emission 400- 460 nm).

Visualization of actin and microtubules was achieved by indirect immunofluorescence microscopy with actin and tubulin antibodies (adapted from Grossmann et al. 2001, Kaminskyj et al. 1994). Leaf segments of 5x5 mm size were fixed in 4 % formaldehyde in 25 mM piperazine-*N, N*′-bis (2-ethanesulfonic acid) buffer (PIPES, pH 6.8) with 2 mM EGTA, 2 mM $MgCl_2$ and 0.05 % Tween 20 (w/v), at room temperature, for 40 min. After being washed in 25 mM PIPES and in 25 mM phosphate buffer (PB, 4.0 g NaCl, 0.1 g KCl, 0.7 g Na_2HPO_4 $2H_2O$, 0.1 g KH_2PO_4 in 500 mL water, pH 6.8), the leaf segments were rinsed in 25 mM BB, pH 6.5. Then, they were transferred to a solution containing 10 mg mL^{-1} driselase, 10mg mL^{-1} chitinase, 16 mg mL^{-1} β-D-glucanase (enzymes provided by InterSpex Products, Inc, San Mateo, CA) and 1 mg mL^{-1} bovin serum albumin (Sigma, Steinheim, Germany) dissolved in 25 mM PB, pH 6.5, at the room temperature, for 15 min. After being rinsed in 25 mM PB, pH 6.5, they were further treated with 0.5 % Triton X-100 in 25 mM PB, pH 6.8, for 10 min. The specimens were washed with 25 mM PB, pH 6.8 then with TB buffer (50 mM Tris-HCl, 150

mM NaCl, pH 7.6). Following a rinse cycle, leaf segments were incubated with a monoclonal mouse anti-actin antibody (Clone C4, ICN BioMed, Inc, Aurora, OH) for the actin localization or with an anti-tubulin antibody (NeoMarkers Fremont, CA) for the tubulin localization, dilued 1:100 (w/v) in antibody diluent solution (DAKO, Carpinteria, CA), at room temperature, for 30 min. Vacuum infiltration was repeated three times for 20 s at 25 mm Hg to promote the infiltration. The specimens were washed two times in TB before incubation in goat anti-mouse IgG Alexa Fluor 488® (Molecular Probes, Leiden, The netherlands) dilued 1:100 (w/v) in antibody diluent, at room temperature, for 1 h. Vacuum infiltration was repeated as described above. Finally, the leaves were rinsed with TB, pH 7.6. The specimens were mounted in TB, pH 7.6 on glass slides and observed by CLSM (excitation 488 nm and emission 505- 540 nm). Confocal fluorescence images were recorded on a multichannel TCS SP2 confocal system (Leica Microsystems, Bensheim, Germany).

Cryofixation and low temperature scanning electron microscope (LTSEM)

For the observation by LTSEM 6 x 5 mm pieces of leaf tissue were cut. Prior to cryofixation, samples were mounted on SEM-stubs using a low-temperature mounting medium and rapidely frozen by plunging them into liquid nitrogen. After cryo-fixation, the samples were placed on a Balzers specimen table under liquid nitrogen. Using a manipulator equipped with an anti-contamination cup, the table was transferred under nitrogen gas flow conditions to a Balzers SCU 020 cryopreparation unit attached to a Jeol JSM 6300. The samples were carefully warmed to – 80 °C and kept there under high-vacuum conditions. Prior to planar magnetron (PM) sputter coating with 20 nm of gold the total pressure was raised to 2 Pa with pure argon gas. After restablishing high-vacuum conditions the coated samples were transferred on-line into the SEM, observed and photographed at – 150 °C using an accelerating voltage of 10 kV.

Results

Quantification of the metrafenone effect on *B. graminis*

Evaluation of the preventive activity

The evaluation of one day preventive activity of metrafenone on *B. graminis* in wheat and barley was carried out at 24, 36 h and 7 d post inoculation (hpi, dpi). At 24 hpi corresponding to 2 days post application (dpa), we investigated the frequencies of germination of *Bgt* and *Bgh*. Metrafenone significantly reduced fungal germination compared to the untreated leaves. At the low concentration of 4 ppm, the frequency of germination decreased by around 30%. At 36 hpi, the frequencies of appressoria with one lobe or more than two lobes, the latter being indicative for unsuccessful penetration, and elongated secondary hyphae of *Bgh* were evaluated. At the concentration of 4 ppm, the preventive metrafenone treatment increased the proportion of conidia forming multilobed appressoria by 30% and lowered the frequency of elongated secondary hyphae by 25%. Moreover, metrafenone enhanced the percentage of appressoria giving rise to non-penetrated papillae while reducing the proportion that succeed in forming a haustorium. Interestingly, the treatment of the fungal inoculum instead of the leaves resulted in the same situation that was already observed after one-day preventive treatment of the leaves, with the difference that the reduction of germination was stronger. At 7 dpi, one-day metrafenone preventive activity on mycelium formation and sporulation of *Bgt* was examined in wheat (Figure 1). The average reduction in mycelium compared with the untreated control corresponded to reductions of 85% to 98% at 7 dpi, for the concentrations of 0.004 and 1 ppm, respectively. Thus, metrafenone effectively prevented wheat from powdery mildew fungus invasion by reducing fungal germination and inhibition of fungal development after the appressoria formation.

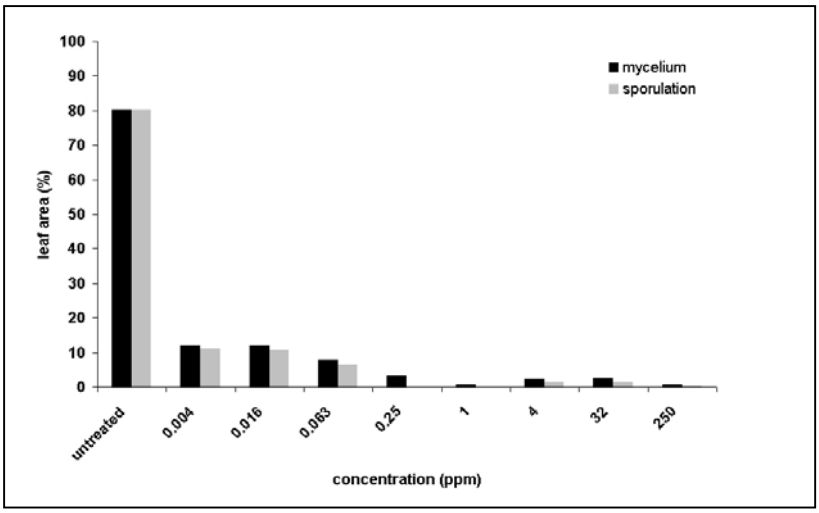

Figure 1. Effect of metrafenone on development of *Blumeria graminis* f.sp. *tritici* on wheat at 7 days after inoculation, following treatment one day before inoculation. Proportion of mycelium and sporulation were evaluated by fluorescence microscopy after Uvitex 2 B staining.

Evaluation of eradicative activity

Metrafenone was directly applied on inoculated wheat leaves at 8 hpi and 2 dpi. Subsequently, mycelium formation and sporulation frequencies were assessed at 7 and 14 dpi. The figures 2A and 2B show the evaluation of metrafenone treatment at 8 hpi, when conidia had germinated. Concentrations of 0.063 ppm metrafenone and higher significantly decreased mycelium formation. At 4 ppm, metrafenone reduced mycelium formation by 93% and 96% at 7 and 14 dpi, respectively. The efficiency of metrafenone at very low concentrations was remarkable. Figures 3A and 3B show that metrafenone treatment at 2 dpi, when haustoria were fully developed, was less efficient. At the concentration of 4 ppm, mycelium formation was reduced by 55% and 62% at 7 and 14 dpi, respectively. Although mycelium was detected on the leaves, the Uvitex 2B staining could not clearly distinguish between live and dead hyphae. Hence, evaluation of the hyphal cell death after metrafenone application was achieved by phloxine B staining, at 4 dpi. At the concentration of 4 ppm, phloxine B staining revealed hyphal cell death as early as 1 h post application and about 60% of mycelium

proportion showed cell death at 12 h post application. Hence, though mycelium was observed after treatment, fungi were usually dead. Together, metrafenone had a significant effect on fungal survival and propagation.

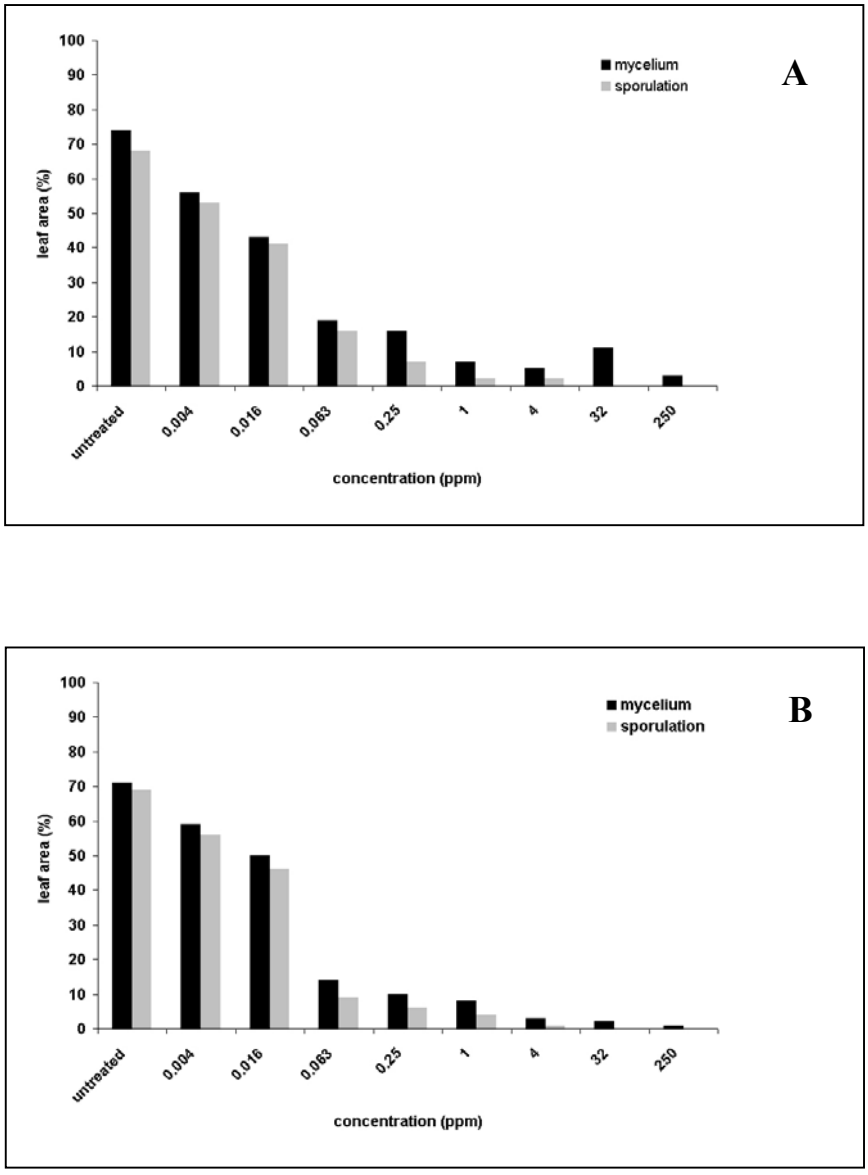

Figure 2. Effect of metrafenone on development of *Blumeria graminis* f.sp. *tritici* on wheat at 7 days (A) and 14 days (B) after inoculation. (Treatment 8 hours after inoculation). Proportion of mycelium and sporulation were evaluated by fluorescence microscopy after Uvitex 2 B staining.

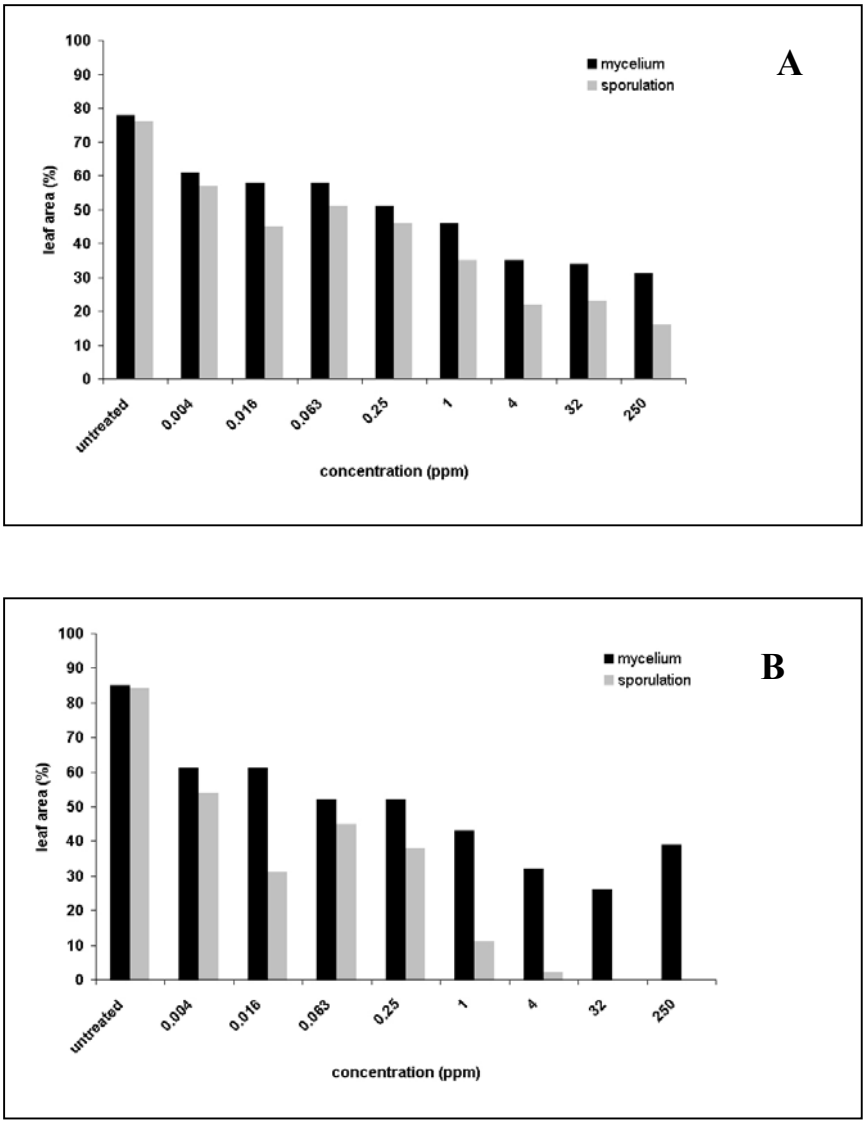

Figure 3. Effect of metrafenone on development of *Blumeria graminis* f.sp. *tritici* on wheat at 7 days (A) and 14 days (B) after inoculation. (Treatment 2 days after inoculation). Proportion of mycelium and sporulation were evaluated by fluorescence microscopy after Uvitex 2 B staining.

Evaluation of the curative activity

Figures 3A and 3B demonstrate that metrafenone treatment at 2 dpi efficiently reduced sporulation. At the concentration of 4 ppm, sporulation decreased by 71% and 98%, at 7 dpi and 14 dpi, respectively. Concurrently, the comparison of the proportion of leaf surface with fungal mycelium to that of sporulation showed a difference of 1% and 2% in untreated leaves

and 13% and 30% in metrafenone-treated leaves, at 7 dpi and 14 dpi, respectively. This indicated that even if mycelium partially developed, sporulation was affected by low concentrations of metrafenone. Often, conidiophore formation was not observed although mycelium was present. Notably, there was a significant reduction of sporulation between 7 and 14 dpi. These data provided evidence that metrafenone inhibited sporulation and showed curative activity. It appeared that the conidiophores showed also a rapid cell death after metrafenone treatment.

Morphological analysis of the metrafenone effect on *B. graminis*

Metrafenone effect on germling morphogenesis

To examine whether metrafenone affected the early development of conidia of *Bgt* and *Bgh*, 4 ppm metrafenone was applied on the first leaf segments one day before inoculation. At 1 dpi, fungal germlings were observed by low temperature scanning microscopy.

On untreated leaves, the appressoria formed a typical apical hook-shaped lobe (Figure 4a) from which a penetration peg was produced. When attempted penetration from the first lobe failed, a second was formed behind the first. On the metrafenone-treated leaves, the fungicide caused a reduction of conidia germination, and where conidia germinated, subsequent appressoria frequently presented two or three lobes (Figure 4b). The appressoria were functional in the sense that they could form infection pegs but penetration was generally blocked in host cell wall papillae. The fungus rarely developed a haustorium and elongated secondary hyphae. If the germling established a haustorium, it was frequently malformed and encapsulated, and consequently, the pathogen stopped to grow.

Direct effect of metrafenone on the fungal morphogenesis

To investigate the direct effect on *Bgt* and *Bgh*, the fungicide was applied on the inoculated leaves at 8 hpi, 2 dpi and 4 dpi. Fungal development was observed by low temperature scanning microscopy.

In non-treated controls, three days after inoculation, the colonies consisted of a conidium with the primary and the secondary germ-tube, primary appressorium and numerous hyphae (Figure 4c). The tip-growing cells comprise a cylindrical shank of constant diameter and an apical dome (Figure 4d). From the superficial hyphae, secondary appressoria were formed with small globular structures disposed alone or in pairs on opposite sides of the hyphae (Figure 4e). Four to five days after inoculation, leaves showed extensive colonies with dense surface of mycelium and numerous conidiophores consisting of a mother cell producing chains of conidia separated by regularly spaced septa (Figure 4f).

Metrafenone caused a fungal growth delay, a number of morphological anomalies and rapid collapse of mycelium (Figure 4g). The diameter of treated hyphae was larger than that of untreated hyphae. The form of the apices changed from cylindrical to spherical and the tip of the apical cells became swollen often followed by a burst and release of globular structures (Figure 4h). These globules were stainable with acridine orange but not with Uvitex 2B. Therefore, they represented most likely fungal cytoplasm and not cell wall material. Hyphae with ruptured hyphal tips were often collapsed. Swelling and bursting were occasionally seen in subapical regions or near secondary appressoria. Furthermore, several hyphal tips were bifurcated (Figure 4i). Secondary appressoria were more abundant than normal and grouped closely together (Figure 4j). They were also more round or bifurcated.

Additionally, when inoculated leaves were treated with metrafenone at 4 dpi, sporulation was delayed or inhibited by metrafenone. Concurrently, metrafenone caused formation of aberrant conidiophores. The conidiophores formed elongated tubes of uniform diameter (Figure 4k) or

a chain of conidiospores with irregular or lacking septation. Irregular septation was also observed in surface hyphae.

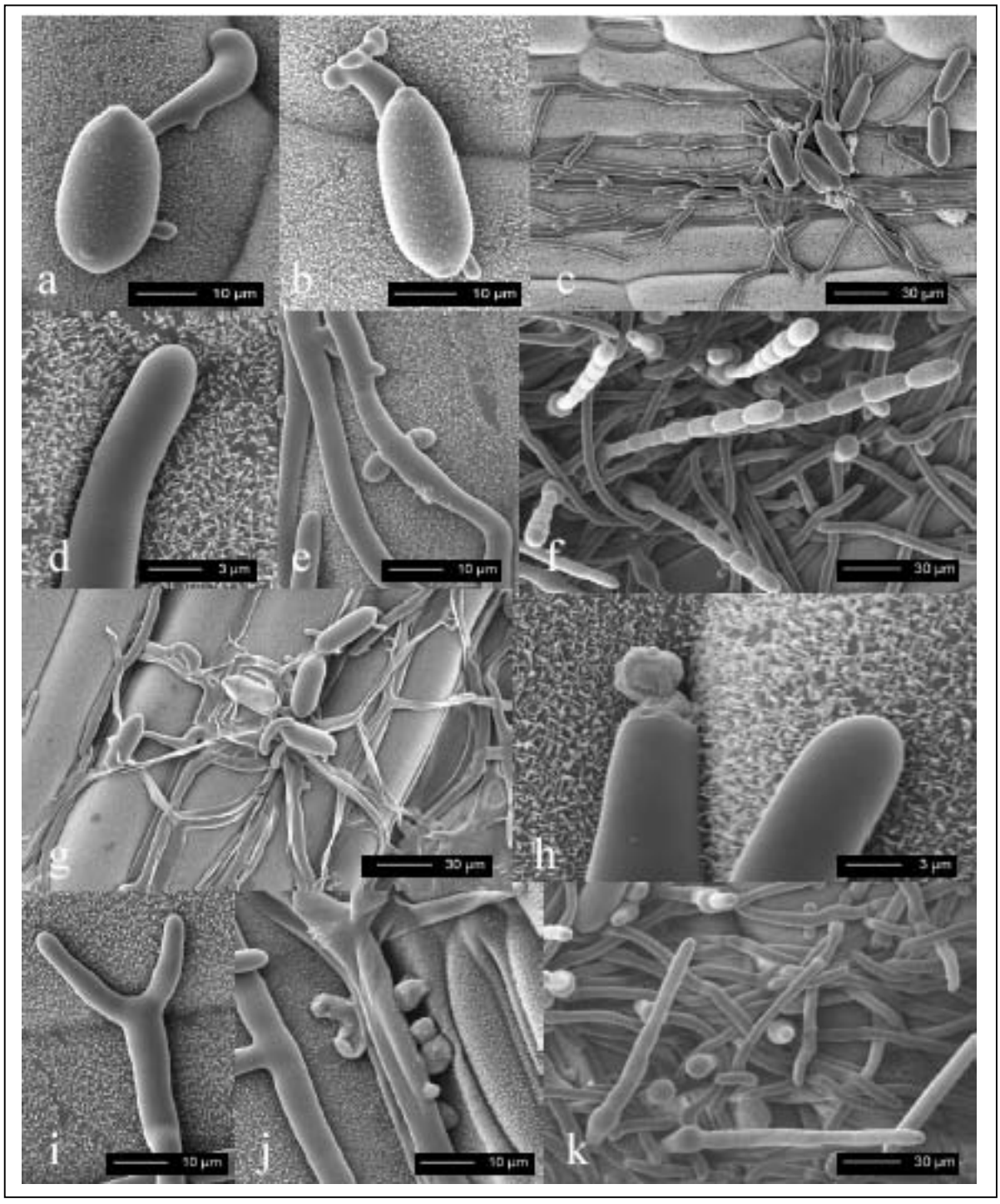

Figure 4. LTSEM of *Blumeria graminis* f.sp. *hordei* on barley leaves after treatment with metrafenone (4ppm). Untreated leaves were included as a control. Conidia formed one lobe (arrow) in the control (a) and was malformed and multilobed (arrows) after preventive treatment (b). In the control, colony consisted of conidium with numerous hyphae (c), that showed normal tips (d) and secondary appressoria (arrows) (e); and conidiophores that produced a chain of conidia separated by regularly spaced septa (f). After curative treatment, the mycelium collapsed (g), the hyphal tips were swollen and extruded globule of fungal cytoplasm (arrow) (h), or bifurcated (i), the secondary

appressoria were abundant (arrow), closely spaced and frequently bifurcated (j), the conidiophores were often tubular (arrow) (k).

Histochemical analysis of metrafenone effect on *B. graminis*

Conventional fluorescence microscopy and confocal laser microscopy were used to analyze the distribution of different components involved in polar growth in *B. graminis*, to control whether metrafenone could affect them.

The cell walls was stained with congo red. In untreated fungi, congo-red stained hyphal tips more brightly compared to subapical cell wall regions indicating areas of polarized cell growth (Figure 5a). In metrafenone-treated hyphae, staining was weaker in the hyphal tips and instead irregularly distributed along the hyphal walls (Figure 5b). This indicated a defect in polarized cell growth and a loss of cell polarity particularly in those hyphal tips that had become spherical. In parallel, we compared metrafenone effect to that of polyoxin D, which caused a very strong swelling of the hyphal tip followed by bursting (data not shown). However, other typical effects of metrafenone were not observed after polyoxin D treatment of *Bgh*.

In living hyphae, apical vesicles were stained with FM4-64. In the control, FM4-64 revealed an accumulation of vesicles at the extreme hyphal tip (Figure 5c), whereas such vesicles were delocalized from the apex, after metrafenone application (Figure 5d). As described already by Dijksterhuis (2003), FM4-64 stains also vesicles within the “Spitzenköper”. Hence, the loss of cell polarity could be well be caused by a disturbance of the “Spitzenköper” by metrafenone.

Furthermore, cellular patterns of actin and tubulin were visualized by indirect immunofluorescence (IIF) microscopy. In control fungi, anti-actin antibodies revealed typical actin patches at tips of appressoria, hyphae (Figure 5e), branches, and immature conidiophores (Figure 5k). Actin formed a peripheral cap in the hyphal apex. Filamentous

actin, as usually stained with phalloidin-coupled dyes, is not highlighted by the anti-actin antibodies. In conidiophores, actin fluorescence occurred also at the site of septum formation. In metrafenone treated fungi, we discovered a change in actin organization compared to the control. IIF microscopy showed a delocalisation of actin away from the apex to the subapical region of hyphae (Figure 5f), branches and immature conidiophores (Figure 5l). Actin fluorescence was undetectable in swollen hyphal tips and sites of septum formation, as well. Simultaneously, the effect of metrafenone on fungal morphogenesis was compared to that of cytochalasin D. Cytochalasin D led to very similar morphological anomalies and actin delocalization observed after metrafenone treatment (data not shown). To test if metrafenone directly inhibits actin polymerization, an actin polymerization assay for metrafenone and cytochalasin A was carried out. This test indicated that metrafenone does not inhibit actin polymerization *in vitro* and thus the actin cytoskeleton was likely indirectly affected by metrafenone (data not shown).

In controls, anti-tubulin antibodies marked a longitudinal pattern of microtubule in appressoria, hyphae (Figure 5g) and conidiophores (Figure 5i). After metrafenone treatment, longitudinal microtubules were mostly observed in subapical region of hyphae (Figure 5h) and conidiophores (Figure 5j and 5o). However, the microtubules were often replaced by a "star-bursting-like pattern" (Bachewich and Heath, 1998) in hyphae and conidiophores. The effect of metrafenone on the microtubules was compared to benomyl. Benomyl induced disruption of microtubules and looping of the hyphal tips (data not shown). Together, microtubules seemed to be not directly affected by metrafenone.

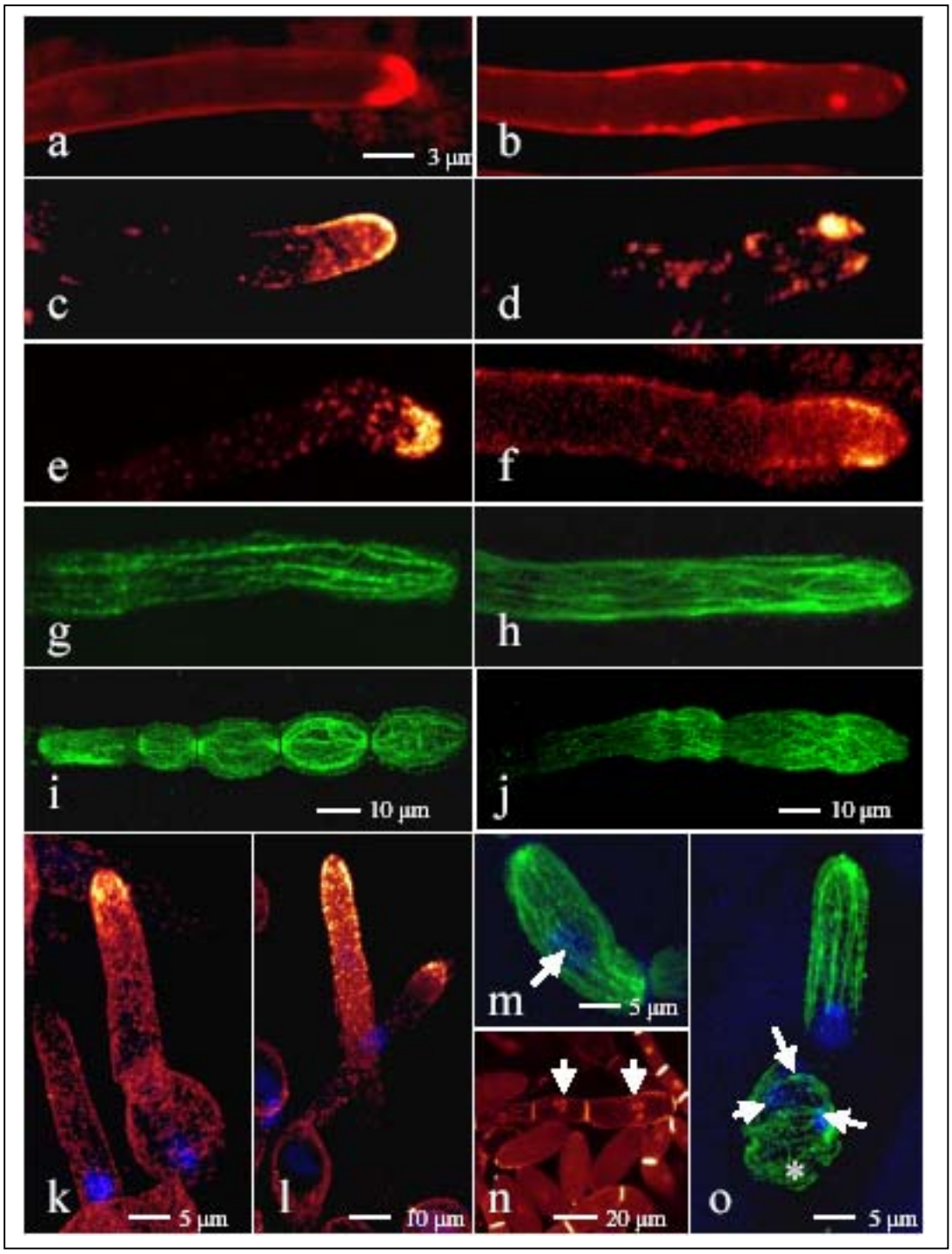

Figure 5. CLSM micrographs showing cytological features of *Blumeria graminis* f.sp. *hordei* on barley leaves, in untreated leaves (a, c, e, g, i, k, m) and after treament with metrafenone (4ppm) (b, d, f, h, j, l, n, o). The hyphal tip (a-h) and the the conidiophore (i-o) were observed by confocal scanning laser microscopy. The distribution of glucan was stain by congo red (a,b), the vesicles by FM4-64 (c,d). The actin was localized after staining with anti-actin antibodies (e,f,k,l). Visualization of tubulin was localized after staining with anti-tubulin antibodies (g, h, i, j, m, o). Distribution of nuclei (arrows) was observed with the localisation of actin after staining with hoechst and anti-actin antibodies (k-l), with the localisation of tubulin after staining with hoechst and anti-tubulin antibodies (m, o) and with the

visualization of the septa after staining with Uvitex 2 B and Hoechst (n). The mother cell is indicated by a star.

Finally, the position of nuclei in treated fungi was also different from that of untreated control. Hoechst 33342 staining showed that in the control, the conidiophores were normally composed of a uninucleate mother cell (Figure 5k) producing chains of uninucleate conidiospores (Figure 5m). After fungicide treatment, some mother cells were multinucleated (Figure 5o) and the condiospores, that were showed irregularly spaced septa, contained several nuclei or nuclear fragments (Figure 5n). As well, Hoechst staining revealed proliferation or fragmentation of nuclei in the regions of hyphal swelling, bifurcation and at abundant secondary appressoria (not shown).

Discussion

Treatment of barley and wheat with metrafenone resulted in effective protection against *B. graminis*. The preventive treatments caused a significant reduction (around 30%) in germination of both *Bgh* and *Bgt* and blocked development beyond the primary appressoria. On metrafenone-treated leaves, appressoria typically formed 2 or 3 lobes. Similar multilobed appressoria appeared on resistant host cultivars in case of unsuccessful penetration by the apical lobe (Carver, 1986). Metrafenone enhanced the number of appressoria giving rise to papillae whilst reducing the proportion that succeeded in forming a haustorium. Additionally, treatment of the conidiospores used for the inoculation involved also an increasing of multilobed appressoria and reduction germination. Since inoculum treatment and leaf treatment had similar effects, it was concluded that metrafenone exerts a direct effect on the fungus and likely did not induce host resistance. The fungicide might rather delay fungal development, giving the host more time to prevent penetration. This could explain the

enhancement of the number of papillae and multilobed appressoria. It is remarkable that the actin was delocalized in the apressorial lobe indicating that the fungicide affected polar growth of the penetration organ. In *Magnaporthe grisea*, polarization of actin cytoskeleton to the site of penetration-hypha formation was suggested to be involved in localized wall modification, which is essential for penetration (Bourett and Howard, 1992; Howard 1994, Xu et al., 1998). Together, the fungicide might cause disturbance of the peg cell polarity and prevent successful fungal penetration of the host cell.

When metrafenone was applied directly on *B. graminis*, the mycelium proportion was greatly reduced compared with controls, for the treatment at 8 hpi and 2 dpi. Metrafenone is an efficient eradicative fungicide at very low concentrations, if it is applied early enough after infection (earlier than 2 days after inoculation). Moreover, metrafenone has also a significant effect on the fungal survival. Mycelium cell death occurred as early as 1 to 3 h after metrafenone application. The reduction of mycelium formation was correlated with rapid collapse of hyphae. Hyphal collapse was associated with the swelling and bursting of hyphal tips. Burst hyphal tips released globule of cytoplasm. The cytological analysis indicated that the swelling of hyphal tips could be due to weakening of cell wall at the apex, disturbance of apical vesicles delivery and disruption of F-actin-cap at the apex. These results indicate a compromised polar growth and loss of cell polarity. It has been reported that cytochalasin A or latrunculin B treatment results in swelling of hyphal tips, with disruption of actin, disturbed vesicle delivery and irregular wall deposition at the hyphal tips (Bachewich and Heath, 1998; Heath et al., 2003; Torralba et al., 1998). However, although the effect of cytochalasin D on *B graminis* was very similar to that of metrafenone, an in vitro actin polymerization assays indicated that metrafenone did not directly inhibit actin polymerization suggesting that the chemical rather induced the rearrangement of the actin pattern. Proteins that play an important role in regulating the organization of the actin include the Ras and Rho GTPases including

Rho, Cdc42 and Rac (Tanaka and Takai, 1998; Momany, 2002; Harris and Momany, 2004). In *Ashbya gossypii*, the mutant of *Agrho3* showed swelling with delocalized actin at the hyphal tips (Wendland and Philippsen, 2001). In *Colletotricum trifolii*, the mutational activation of *Ras* gene exhibited hyphal tips tending to burst (Truesdell et al.,1999).

Moreover, metrafenone frequently caused bifurcation of hyphal tips and secondary appressoria, likely indicating the loss of cell polarity. A mutation in the *Neurospora crassa* actin gene results in branching of hyphal tips and alteration of actin at the tip (Virag and Griffiths, 2004). In a temperature-sensitive mutant of *Aspergillus niger*, apical branching involves dislocation and disappearance of the Spitzenkörper (Reynaga-Pena and Bartnicki-Garcia, 1997). The process of apical branching was suggested to be due to shift of vesicles from the tip to the side initializing the formation of new hyphal outgrowths (Raudaskoski et al., 1994). Similarly, metrafenone might cause the bifurcation by delocalization of actin, vesicles and Spitzenkörper at hyphal tips. Thus, in metrafenone-treated hyphae, the defects in apical polarized growth and cell polarity could be a consequence of the failure in maintaining the polarity of the actin cytoskeleton.

Additionally, metrafenone treatment resulted in abundant secondary appressoria grouped together in abnormal close distance. The high number of lateral branches was also observed in hyphae of *Saprolegnia ferax* after application and consecutive removal of latrunculin B. The phenomenon was preceded by formation of radial arrays of actin in regions without detectable surface protrusion. These sites were consistent with future branches (Bachewich and Heath, 1998). Accordingly, in the absence of focused actin at the apex, hyphal growth might be not directed and thus cells grew in multiple directions. In the metrafenone-treated hyphae, multinucleate cells and irregular septation were observed as well. In filamentous fungi, actin has been localized at the sites of septum formation and cytochalasin A was able to block septum formation in *A. nidulans* (Torralba et al., 1998; Harris et al., 1994). Interestingly,

Westfall and Momany (2002) have reported that mutation of septin *AspA* shows irregular septa and hyperbraching in *A. nidulans*. Localization of AspB at the septation site is dependent on actin. Metrafenone could prevent the formation of septation and induce hyperbranching by disturbing the actin cytoskeleton. The hyperbranching possibly revealed multiple randomly distributed initiation sites of polar growth indicating defects in establishment of cell polarity.

Furthermore, microtubules were reported to be implicated in polarized growth and vesicle transport (Harris and Momany, 2004; Pedregosa et al., 1995). However, IIF microscopy has revealed no disruption of microtubules by metrafenone. Microtubules were often replaced by a "star-bursting-like pattern" in hyphae. This reorganization of microtubule was also observed in *S. ferax* after disruption of actin and, therefore, might be a secondary effect of actin defects (Bachewich and Heath, 1998).

Finally, metrafenone inhibited and delayed the sporulation. A metrafenone treatment at 2 dpi reduced sporulation by 98%, at 14 dpi. Hence, metrafenone has a curative activity The inhibition of sporulation was associated with the malformation of conidiophores, that showed multinucleate cells and irregular septation. This suggests that mitosis proceeded without septum formation, indicating that cytokinesis was impaired. Again, actin was delocalized in the tip of the young conidiophores and actin associated with septa was hardly detectable after metrafenone treatment. Actin is known to play a central role in septum formation and be involved in cytokinesis (Harris and Hamer, 1995). The effect of metrafenone on *B. graminis* shows interesting similarities with the deletion of *cflB*, the *Penicillium marneffei* RAC homolog. Δ *cfl B* results in cell division and growth defects in conidiophores such that cells become multinucleate, exhibit inappropriate septation (Boyce et al., 2003). This further supports the idea that the fungicide might cause irregular septation and division of nuclei by disturbance of actin organization.

These results taken together suggest that the mode of action of metrafenone and its potential target are involved in hyphal morphogenesis, polarized hyphal growth, and the establishing and the maintenance of cell polarity in *B. graminis* in wheat and barley. The morphological anomalies induced by the fungicide might be the consequence of the failure to polarize the actin cytoskeleton. Metrafenone might interfere with processes that are essential to establish and maintain cell polarity, which depends on proper polar actin organization.

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Studies on the mode of action of Metrafenone, a new systemic fungicide compound

Krystina Opalski[1], Guido Albert [2], Ralph Hückelhoven[1], Karl-Heinz Kogel[1]

[1)] Institute of Phytopathology and Applied Zoology, Justus-Liebig-University Giessen, Heinrich-Buff Ring 26-32, D-35392 Giessen, Germany

[2)] LPP, Mainz, Germany

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Abstract

Powdery mildew fungi are among the major pathogens causing diseases of cereals in the world. The mode of action of a novel systemic benzophenone-derivated fungicide, metrafenone has been analyzed on the powdery mildew fungi of barley (*Blumeria graminis* f. sp. *hordei*) and wheat (*Blumeria graminis* f. sp. *tritii*). We have evaluated the preventive and curative treatment of metrafenone with other systemic fungicides, investigated the effect of metrafenone on fungal morphogenesis and mimicked the effects of fungicide with specific inhibitors. Metrafenone inhibits the formation and development of different stages of the cycle of powdery mildew fungi. The development of the pathogen was blocked by inhibition of infection structures, mycelium formation and sporulation. Metrafenone offer a exceptional combination of protective, eradicative and curative performance. Metrafenone was more efficient than kresoxim-methyl, spiroxamine and quinoxyfen, with low concentration, when it was applied earlier than 2 days after inoculation. After preventive treatment, metrafenone led to an increase of multilobed non-functional appressoria. After curative treatment, the fungicide induced swelling, bursting and collapse of hyphal tips. Secondary appressoria and hyphal tips were also frequently bifurcated. Hyphal branching increased whilst spacing between branches was closer than normal. Conidiophores had a tubular shape without septation or formed a chain of conidia with irregular septation. The mode of action of metrafenone strongly differs from so far known mechanisms and the effect of fungicide was similar to that of cytochalasin D, an inhibitor of actin polymerization.

Introduction

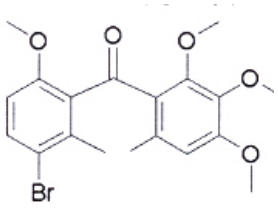
Powdery mildew of cereals is caused by an ascomycete, obligate biotrophic fungus *Blumeria graminis* ff. spp., an obligate parasite. The disease is widely distributed on cereals throughout the world; it is most damaging in cool, wet climates but occurs even in semiarid areas.

Powdery mildew fungi produce all vegetative and reproductive organs expect haustoria on the surface of their host plants and indirectly damage the plant by reducing photosynthesis and increasing transpiration and respiration. The most important cereals in Europe are wheat and barley, and the major measures of disease control are the use of genes conferring resistance in the host, and the use of fungicides. Prior to the 1960s, protectants and multi-site inhibitors fungicides could control powdery mildews. However, they were used with high dose rates, and their cost was such that applications were only economic on high value vegetable crops. In the early 1970s, the situation changed dramatically with the introduction of the first successful systemic product, benomyl, that established new standards of control for powdery mildews. The mode of action of these systemic involved specific inhibition of physiological processes and they were effective at lower concentrations. However, the impact of chemical control has been very much tempered by the ease of powdery mildews developing resistance, quickly rendering many systemic fungicides ineffective (Hollomon and Wheeler, 1999). For instance, sterol demethylation inhibitors (DMIs) are not used as single compounds against powdery mildew anymore. Likewise, inhibitors of sterol reductase and isomerase, the morpholines, have shown some sensitivity shifts (spiroketalamines are also affected due to cross resistance). Isolates of wheat powdery mildew resistant to both strobilurins and the quinoline fungicides, quinoxyfen were recently detected at a low frequency in some parts of Europe (www.frac.info). Therefore, finding new and effective disease control strategies represents an important challenge to the agrochemical industry. To face problem arising from the development of resistance of *B. graminis*, against the fungicides, BASF industry has provided a novel systemic fungicide, metrafenone (Figure 1). Metrafenone is the first benzophenone-derivated fungicide and represents an active ingredient that has not been used in chemical plant protection until now.

Figure 1.

Chemical name (IUPAC):
(3-bromo-6-methoxy-2-methylphenyl) (2,3,4-trimethoxy-6-methylphenyl)-methanone

Molecular formula: C_{19} H_{21} Br O_5
Molecular mass: 409.27 g/mol
CAS registry number: 220899-03-6



In this current investigation, we have studied the mode of action of metrafenone on powdery mildew fungi of barley (*Blumeria graminis* f. sp. *hordei*, *Bgh*) and of wheat (*Blumeria graminis* f. sp. *tritici, Bgt*). The results show that metrafenone is a fungicide unique in its mode of action, since it inhibited the development of different stages of the life cycle of the powdery mildew fungus. Metrafenone has an exceptional combination of protective, eradicative and curative activities. The efficiency of metrafenone to control powdery mildew was similar or even better when compared to conventional fungicides in use, especially at low concentrations. Metrafenone strongly differs from traditional fungicides, as its action is different to so far known mechanisms. The comparison of metrafenone with specific inhibitors showed that the fungicide had a similar effect when compared to the actin polymerization inhibitor, cytochalasin D.

Materials and Methods

Plant material, pathogen and inoculation

Barley (*Hordeum vulgare* L.), and wheat (T*riticum aestivum* L.) were grown in a growth chamber at 18 °C with 60 % relative humidity and photoperiod of 16h (240 µmol

photons/m^2/s). For microscopy, dense inoculation of first leaves was performed with 50-100 conidia/mm^2 leaf area of *Blumeria graminis* f sp. h*ordei* and *Blumeria graminis* f. sp. *tritici*, at the 7^{th} day after germination. The fungus was cultivated on barley and wheat under the same condition.

We also tested metrafenone on other fungi. *Ashbya gossypii* (culture number 317, provided by Speakman J.B, BASF, Limburgerhof, Germany) was grown on Czapek-Dox-V 8 (CDV8) medium with 15g succrose / litre. *Alternaria alternata* and *Septoria tritici* were grown on PDA (Merck).

Treatment with anti-fungal agents

Metrafenone (BAS 560F, 300 g/l a.i. solo, provided by BASF Aktiengesellshaft, Ludwigshafen, Germany) belongs to the novel benzophenone fungicide group.

We compared preventive, curative and eradicative activities of metrafenone with those of systemic fungicides, which act against powdery mildew: kresoxim-methyl (BAS 490 11 F, 150 g/l a.i. solo), spiroxamine (BAS 9233 1F, Impulse, 700 g/l a. i. Solo), quinoxyfen (BAS 9200 1F, Fortress, 150 g/l a. i. solo). Metrafenone, kresoxim methyl, spiroxamine and quinoxyfen were applied at the concentrations from 0.004 to 250 ppm. Chemicals were prepared with distilled water and applied by spraying to run off with an atomizer, to wheat or barley seedlings. To test the effect of metrafenone on *A. gossypi*, A*lternaria* and *Septoria*, the chemical was directly applied by spraying on Petri dishes.

We tried to mimic the effects of metrafenone by using specific inhibitors: cytochalasin D (0.5µg/ml, Molecular Probes), benomyl (100 ppm, BAS 321 00 F, 500 g/l), polyoxin D (3 ppm, Calbiochem), cyproconazole (25 ppm, ALTO ® 100 SL, 100g/l), nystatin (100 ppm, Sigma), verapamil (250 ppm, Calbiochem), cycloheximide (500 ppm, Sigma), cordecypin (500 ppm, Sigma), actinomycin D (300 ppm, Molecular Probes). All of these chemicals were dissolved in distilled water contaning 0.1% dimethyl sulfoxide (DMSO, Wake). To apply

these chemical, the leaves were immersed in solution-contained the inhibitor and not sprayed because of the high toxicity of these compounds.

Microscopic and macroscopic observations

To visualize the fungi, we used uvitex 2B (Ciba Geigy, Basel, Switzerland), which stains the β-linked cell wall polymers (Wachsmuth, 1988). Uvitex 2B solved in ethyl was directly sprayed on the leaves before the observation by fluorescence microscopy and CLSM (excitation 365 nm and emission 420-460 nm). *Alternaria alternata* and *Septoria tritici* were stained with diethanol and observed by fluorescence microscopy. *Ashbya gossypii* was directly observed by bright field microscopy.

In green house, the rate of infection, measuring the proportion of attacked leaves surface, was evaluated by macroscopic observation.

Results

Evaluation of the metrafenone effect on *B. graminis*

We have analysed protective, eradicative and curative activities of metrafenone on the powdery mildew fungus on wheat. In parallel, we compared the efficiency of metrafenone with that of kresoxim-methyl, spiroxamine and quinoxyfen (Table 1).

Table 1: Fungicide class and mode of action of powdery mildew fungicides.

Fungicide class	Active ingredients	Mode of action	References
Strobilurine	Kresoxim-methyl	inhibits respiration by blocking electron transport through complex III of mitochondrial stops germination and has little curative or eradicative activity	Mansfiel and Wiggins, 1990 Amermann et al., 1992 Margot et al., 1998
Spiroketalamine	Spiroxamine	inhibits sterol 14-15 reductase effect on the step in the sterol biosynthesis pathway stops germination and sporulation	Dutzmann et al., 1996 Tiemann et al., 1997
Quinoline	Quinoxyfen	stops germination in some powdery mildews and appressorium formation in others	Longhurst et al., 1996 Hollomon et al., 1997 Gustafson et al., 1998

Evaluation of the preventive treatment

We compared the preventive effects of metrafenone, kresoxim-methyl, spiroxamine and quinoxyfen on *B. graminis* f. sp. *tritici* (*Bgt*) in wheat. The fungicides were applied at concentrations of 1, 32 and 250 ppm on first leaf segments in Petri dishes. One day after application (dpa), leaves were inoculated. We evaluated the influence of the fungicides on germination at 1 day post inoculation (dpi) and on mycelium formation and sporulation at 7 dpi. We scored the fungal development by fluorescence microscopy after uvitex 2B staining on 4 primary leaf segments, counting 50 conidia each. These experiments were repeated 4 times.

Treatment with 32 ppm metrafenone reduced the germination by 27% when compared with the non-treated control (Figure 2). The percentage of reduction of germination was 99% with kresoxim-methyl, 71% with spiroxamine and 59% with quinoxyfen at the concentration of 32 ppm (Figure 1). Thus, metrafenone inhibited germination but less effective than the other fungicides.

At 7 dpi corresponding to 8 days post application (dpa), we tested preventive activity of fungicides on mycelium formation and sporulation of *Bgt*. The average reduction in mycelium development when compared with the non-treated control was 99% for metrafenone, 93% for spiroxamine, 77% for kresoxym-methyl and 67% for quinoxyfen at 7 dpi, at the concentration of 1 ppm (Figure 3). Hence metrafenone effectively prevented wheat from powdery mildew development by inhibition of mycelium formation. At low concentrations, the protection performance of metrafenone was better than that of the other fungicides.

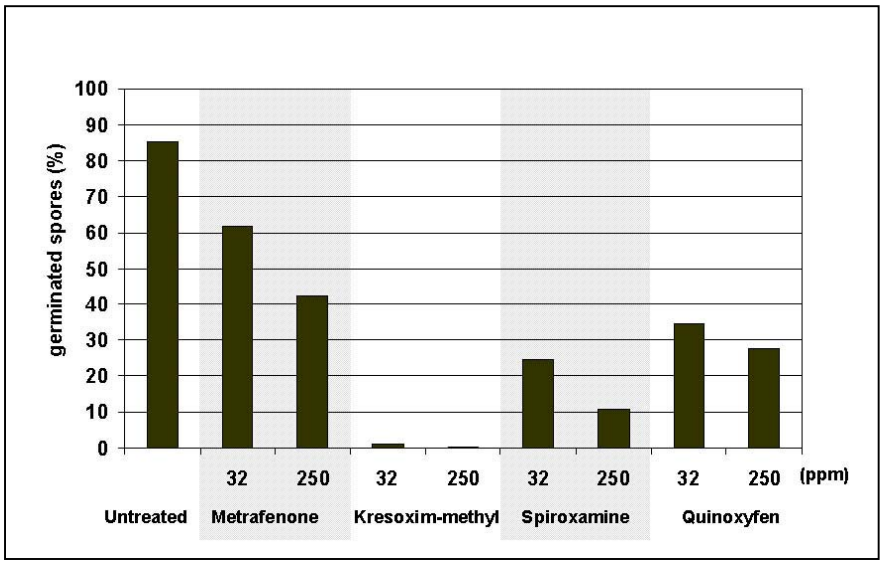


Figure 2. Preventive effect of metrafenone, kresoxim-methyl, spiroxamine and quinoxyfen on germination of *Blumeria graminis* f.sp. *tritici* in wheat at one day after inoculation. Treatment with metrafenone was carried out one day before inoculation. Fungal development was scored by fluorescence microscopy after uvitex 2B staining.

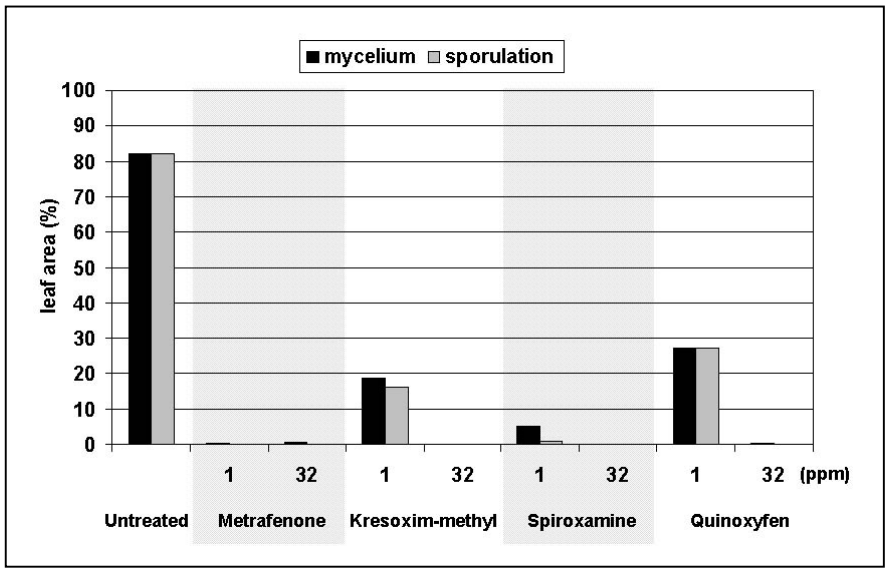


Figure 3. Preventive effect of metrafenone, kresoxim-methyl, spiroxamine and quinoxyfen on development of *Blumeria graminis* f.sp. *tritici* on wheat at 7 days after inoculation (Treatment one day before inoculation). Proportions of mycelium and sporulation covering the leaf surface were evaluated by fluorescence microscopy after uvitex 2B staining.

Evaluation of curative treatment

We evaluated the curative activities of metrafenone, kresoxim-methyl, spiroxamine and quinoxyfen on *Bgt*. The fungicides were applied at concentrations of 1 and 32 ppm on the leaf segments in Petri dishes and whole leaves in greenhouse. In Petri dishes, we studied the influence of the fungicides on mycelium formation and sporulation at 7 dpi. In greenhouse, we evaluated the influence of fungicides on disease severity by macroscopic observation at 7 and 14 dpi. All experiments were repeated 4 times.

Post-inoculation treatment was performed at 8 hpi, when the conidia had germinated. In Petri dishes, the effect on mycelium reduction was 90% for metrafenone, 95% for spiroxamine and 55% for kresoxim-methyl at 7 dpi, at the concentration of 1 ppm (Figure 4). In greenhouse, the reduction of rate of infection, measured as reduction of the proportion of infected leaves surface, corresponded to 79% for metrafenone, 12% for spiroxamine, 58% for kresoxim

methyl, and 10% for quinoxyfen at the concentration of 1 ppm, at 14 dpi (Figure 5). Consequently, metrafenone had an eradicant activity, which was stronger than that of the fungicides, when it was applied at 8 hpi, at a low concentration.

Post-inoculation treatment was also carried out at 2 dpi when haustoria are fully developed. Compared to control, the mycelium formation after treatment with metrafenone was reduced by 37% whereas it was reduced by 60% after treatment with kresoxim-methyl and spiroxamine, (each 1 ppm, at 14 dpi, Figure 6). In comparison with the control, sporulation was reduced by 30% after treatment with metrafenone and kresoxim-methyl, whereas it was reduced by 97% after treatment with spiroxamine (each 1 ppm, at 7 dpi, Figure 6). However, in greenhouse, the reduction of infection was 85% for metrafenone, 40% for kresoxim-methyl and 10% spiroxamine (each 1 ppm, at 14 dpi, Figure 7). In greenhouse, after application of metrafenone or quinoxyfen, a reduction of infection was remarkable between 7 and 14 dpi, whereas the disease recovered in the period after application of kresoxim-methyl and spiroxamine (Figure 7). These results show that the treatment with metrafenone at 2 dpi was too late to fully prevent infection while the kresoxim-methyl and spiroxamine were still effective at high concentrations. Remarkaly, metrafenone inhibited sporulation like kresoxim-methyl and spiroxamine.

Curative treatments with all four fungicides at 4 dpi were too late for full protection. However, after treatment with metrafenone in Petri dishes, sporulation was strongly reduced, although mycelium was formed (Figure 8). After application of metrafenone, in the greenhouse, infection was reduced and did not recover between 7 dpi and 14 dpi. In contrast, after application of kresoxim-methyl and spiroxamine infection recovered after initial reduction (Figure 8). These results show that metrafenone reduced secondary infections by inhibition of sporulation.

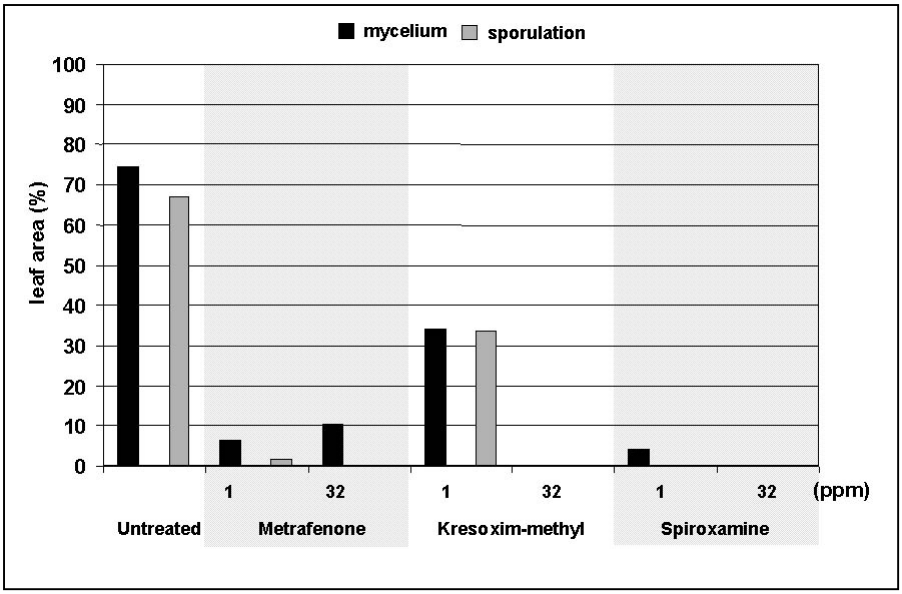


Figure 4. Effect of metrafenone, kresoxim-methyl and spiroxamine on development of *Blumeria graminis* f.sp. *tritici* on wheat at 7 days after inoculation (Treatment 8 hours after inoculation). Proportion of mycelium and sporulation covering the leaf surface were evaluated by fluorescence microscopy after uvitex 2B staining.

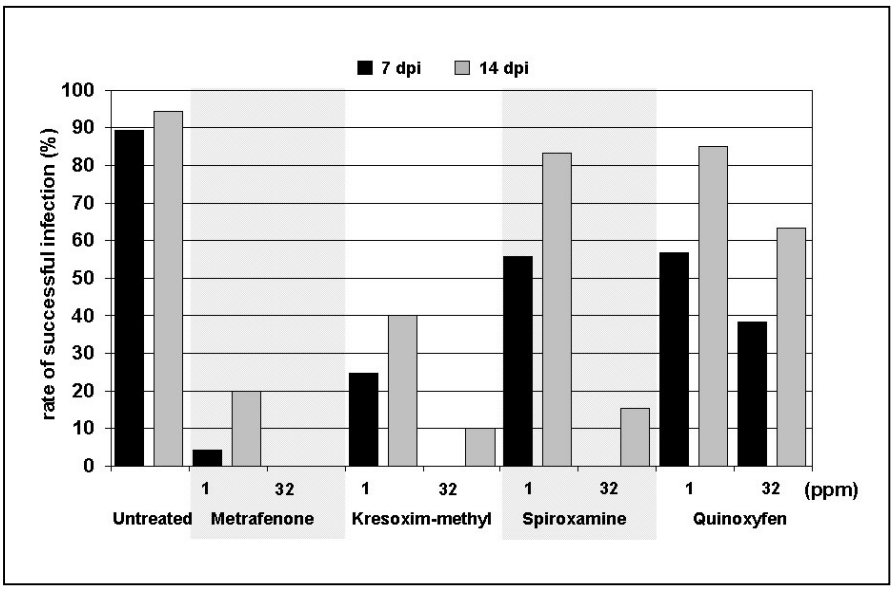


Figure 5. Influence of metrafenone, kresoxim-methyl, spiroxamine and quinoxyfen on disease severity of *Blumeria graminis* f.sp. *tritici* on wheat in greenhouse, at 7 and 14 days, after inoculation (Treatment 8 hours after inoculation). Rate of successful infection was evaluated by macroscopic observation.

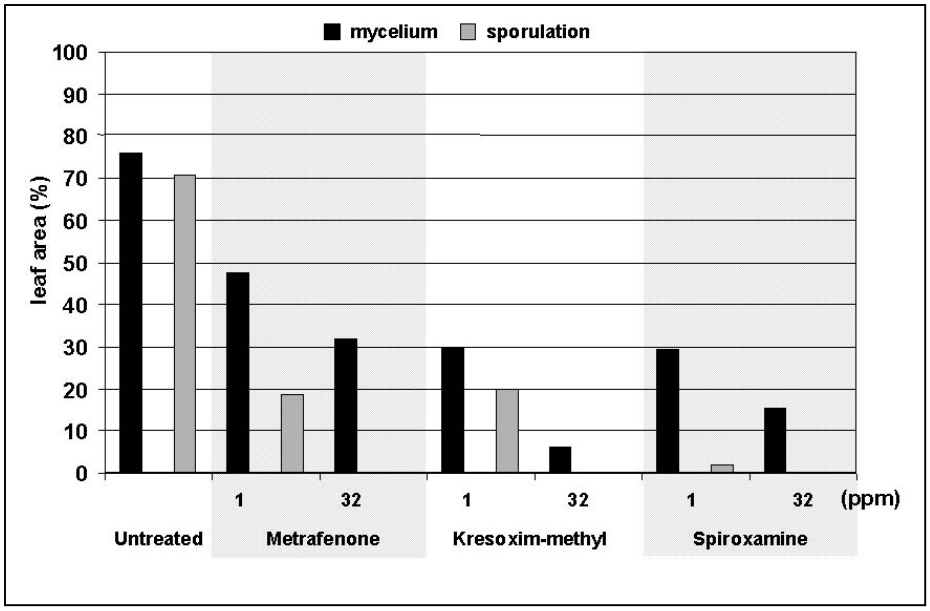


Figure 6. Effect of metrafenone, kresoxim-methyl and spiroxamine on development of *Blumeria graminis* f.sp. *tritici* on wheat at 7 days after inoculation (Treatment 2 days after inoculation). Proportion of mycelium and sporulation covering the leaf surface were evaluated by fluorescence microscopy after diethanol staining

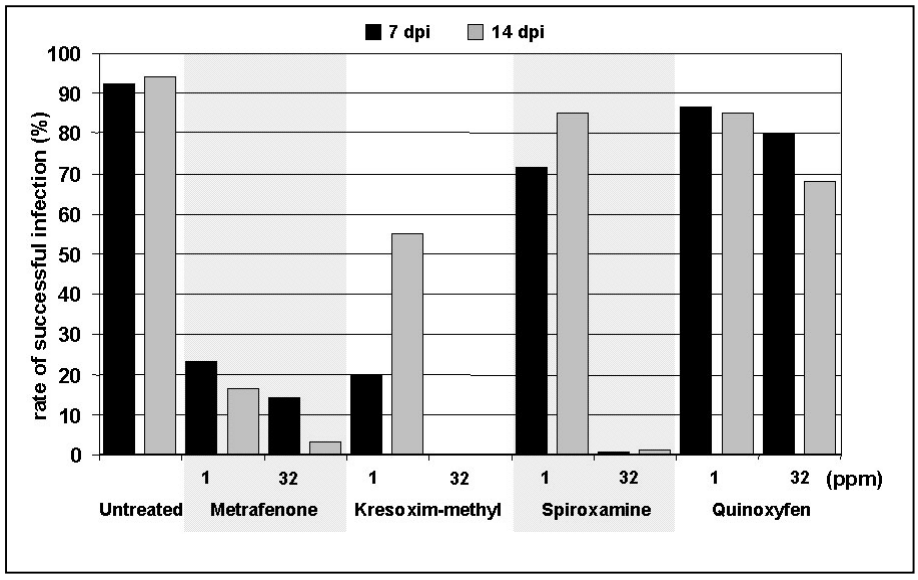


Figure 7. Influence of metrafenone, kresoxim-methyl, spiroxamine and quinoxyfen on disease severity of *Blumeria graminis* f.sp. *tritici* on wheat in greenhouse, at 7 and 14 days, after inoculation (Treatment 2 days after inoculation). Rate of successful infection was evaluated by macroscopic observation.

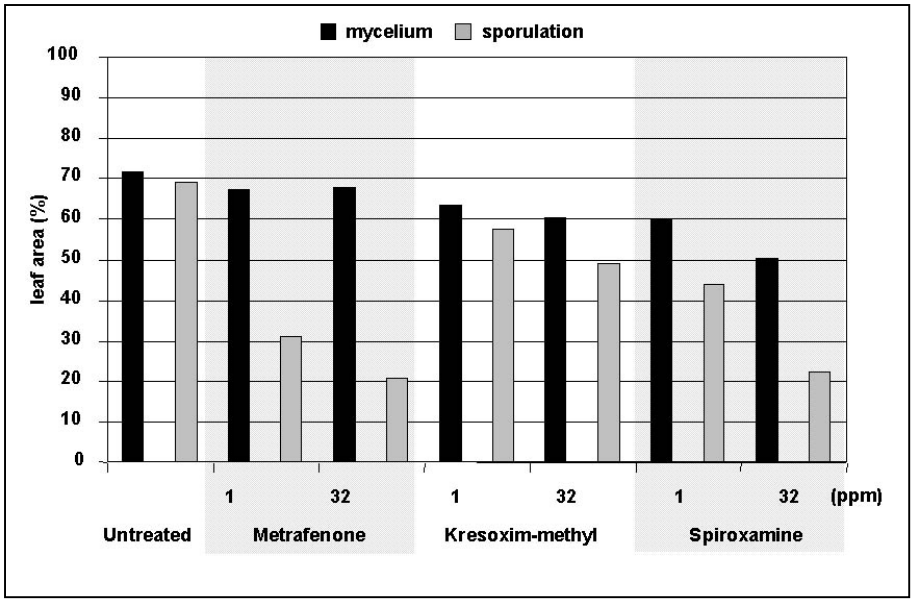


Figure 8. Effect of metrafenone, kresoxim-methyl and spiroxamine on development of *Blumeria graminis* f.sp. *tritici* on wheat at 7 days after inoculation (Treatment 4 days after inoculation). Proportion of mycelium and sporulation covering the leaf surface were evaluated by fluorescence microscopy after uvitex 2B staining

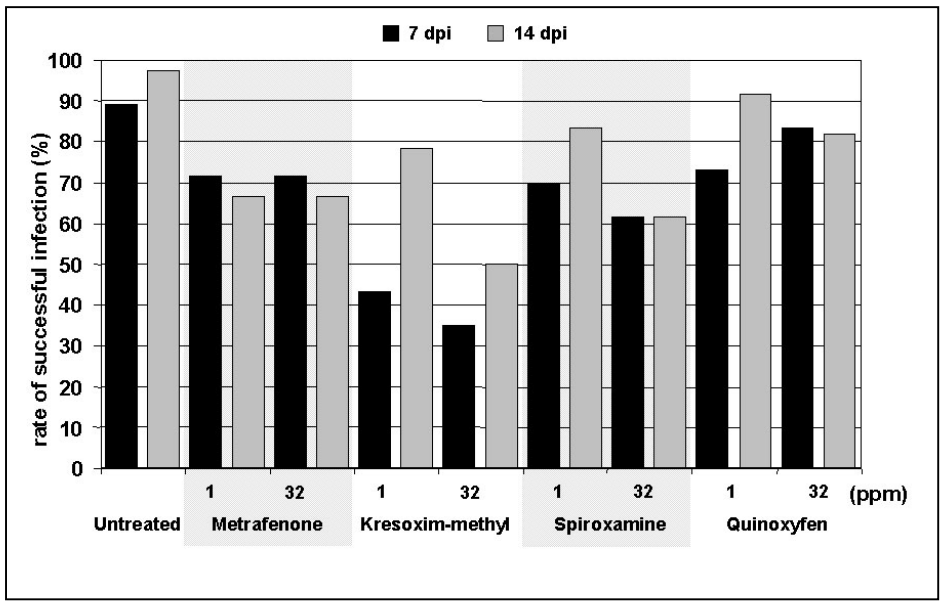


Figure 9. Influence of metrafenone, kresoxim-methyl, spiroxamine and quinoxyfen on disease severity of *Blumeria graminis* f.sp. *tritici* on wheat in greenhouse, at 7 and 14 days, after inoculation (Treatment 4 days after inoculation). Rate of successful infection was evaluated by macroscopic observation.

Study of the effect of antifungal compounds on *B. graminis*

Effect of metrafenone on the morphogenesis of *Bgh*

Although metrafenone is an efficient systemic fungicide to control powdery mildew, the mode of action of metrafenone is still unknown. To search the biochemical target of metrafenone, we investigated the morphological anomalies of *Bgh* after application of fungicide (compare also Opalski et al. submitted). In controls, conidia of *Bgh* formed a primary germ tube after contact with the host surface. Afterwards, a second, germ tube emerged and this mostly differentiated into an appressorium with one lobe, the appressorial hook. Fours days later, conidia had formed numerous hyphae (Figure 10a) with secondary appresoria, that were small globular structures arranged singly or in pairs on opposite sides of the hyphae. Five days after inoculation, leaves showed large colonies with a dense surface mycelium and abundant conidial chains of normal appearance (Figure 10b). After preventive application of metrafenone, conidia of *Bgh* often developed multilobed appressoria. Curative treatment caused swelling and bursting of hyphals tips (Figure 10c). Additionally, secondary appressoria and some hyphal tips were bifurcated (Figure 10d). Secondary appressoria were more abundant than normal and grouped closely together (hyperbranching Figure 10e). Conidiophores had tubular shape with either irregular or missing septation (Figure 10f).

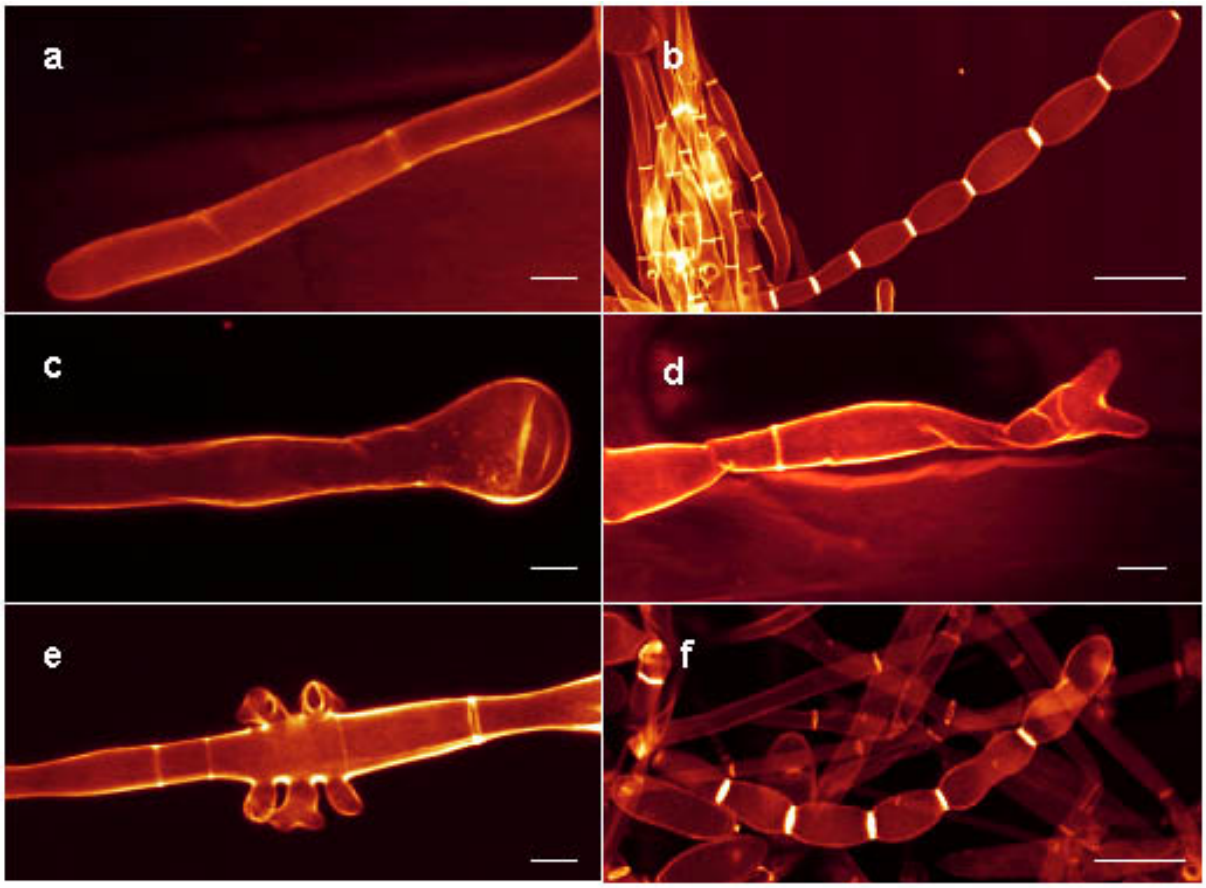


Figure 9. Effect of 1 ppm metrafenone at 5 dpi, 2 dpa on *Bgt* on wheat. In the control, hyphae formed normal hyphal tips (a) and conidiophores with regular septation (b). Metrafenone caused swelling and bursting of hyphal tips (c) bifurcation of the apex (d), hyperbranching (e) and irregular septation of conidiophores (f). Uvitex 2B staining of *Bgt* was observed by CLSM. Bar = 20 µm

Effect of specific inhibitors on the morphogenesis of *Bgh*

To narrow potential targets of metrafenone, we also tried to mimic its effect on the fungus with the specific antifungal inhibitors cytochalasin D, verapamil, polyoxin D, benomyl, nystatin, cyproconazole, cycloheximide, cordecypin, actinomycin D (for their biochemical targets, see the Table 2).

Table 2: List of antifungal chemicals and their effect on *Bgh*.

antifungal compounds	biochemical target	morphological anomalies
metrafenone	unknown	multilobed appressoria swelling and bursting of hyphal tips bifurcation of hyphal tips hyperbranching irregular septation of conidiophores
cytochalasin D	inhibitor of actin polymerization	swelling of hyphal tips bifurcation of hyphal tips hyperbranching irregular septation of conidiophores
verapamil	blocks intracelluar membrane calcium channels	multilobed appressoria swelling of hyphal tips bifurcation of hyphal tips abnormal apical branching
Polyoxin D	inhibitor of chitin synthesis	strong swelling and bursting of hyphal tips subapical swelling irregular septation of conidiophores
Benomyl	inhibitor of tubulin polymerization	swelling of hyphal tips bifurcatin of hyphal tips abnormal apical and subapical branching hyphae curling hyperbranching irregular septation of conidiophores
Nystatin	membrane active antibiotic enhancement permeability to protons	long germ tube swelling of hyphal tips bifurcation of hyphal tips hyphae deformation
Cyproconazole	inhibitor of ergosterol synthesis	multilobed appressoria swelling of hyphal tips followed by hyphal Extension and bursting
Cycloheximide	inhibitor of proteins synthesis	no morphological anomalies
Cordecypin	inhibitor of RNA polymerase	hyphae deformation swelling of hyphal tips
Actinomycin D	DNA synthesis inhibitor	long germ tube hyphae deformation swelling of hyphal tips

Table 2 shows the effect of different specific inhibitors on the morphogenesis of *Bgh*. The common effect for all of chemicals except cycloheximide was swelling of the hyphal tips. However, only treatment with polyoxin D and cyproconazole caused swelling of hyphal tips followed by bursting. Bifurcation of hyphal tips were induced by the treatments with cytochalasin D, benomyl, verapamil and nystatin. Hyperbranching or increase of branching were caused only by cytochalasin D and benomyl. Irregular septation of the conidiophores were induced by cytochalasin D, benomyl and polyoxin D. Although polyoxin D and cyproconazole caused bursting of the hyphal apex, swollen hyphal tips were very big and not similar to the ones observed after treatment with metrafenone. Moreover, polyoxin D and cyproconazole did not induce other morphological anomalies caused by metrafenone. Thus, most similarities with the effect of metrafenone were obtained by cytochalasin D and benomyl treatment. Although the effect of benomyl on *Bgh* was similar to that of metrafenone, benomyl –induced curling of hyphae and an abnormal apical and subapical branching were not observed after treatment with metrafenone.

Consequently, cytochalasin D was the only inhibitor, which has effects strickly similar to that of metrafenone. Cytochalasin D induced in *Bgt* and *Bgh* swelling (Figure 11a) and bifurcation (Figure 11b) of the hyphal tips, hyperbranching (Figure 11c) and irregular septation of the conidiophores (Figure 11d).

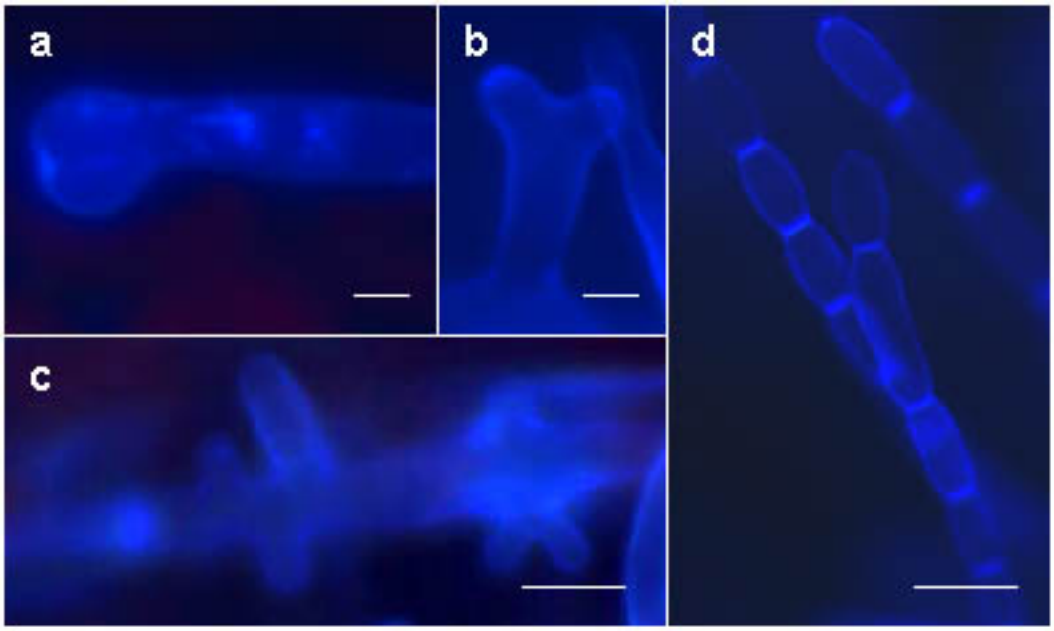


Figure 11. Effect of cytochalasin D at 5 dpi, 1 dpa on *Bgt* on wheat. Cytochalasin D caused swelling (a) and bifurcation (b) of hyphal tips, hyperbranching (c) and irregular septation of conidiophores. Diethanol staining of *Bgt* was observed by fluorescence microscope. Bar = 20 μm

Effect of metrafenone on other fungi

We tested metrafenone on other fungi, like *Ashbya gossypii*, *Alternaria alteruata*, and *Septoria tritici*. At high concentration of 32 and 250 ppm, metrafenone caused swelling of some cells of *A. alteruata* and *S. tritici* and bursting of the hyphal tips of *A. gossypii*. However, after a certain time the fungi continued to grow normally. At low concentration of 4 and 16 ppm, metrafenone did not induce any morphological change of these fungi.

Discussion

Metrafenone inhibited formation and development of the different stages of the life cycle of powdery mildew fungi. Treatment of barley and wheat leaves with metrafenone resulted in effective protection against powdery mildew. After protective treatment, metrafenone

prevented the formation of mycelium more effectively than kresoxim-methyl, spiroxamine and quinoxyfen. The effect of metrafenone on germination was lesser than that of the other fungicides. Curative treatment with metrafenone reduced primary and secondary infection by *B. graminis* by inhibition of mycelium formation and sporulation. It is noteworthy that metrafenone had stronger eradicative and curative activities than kresoxim-methyl, spiroxamine and quinoxyfen, when it was applied early after inoculation (before 2 days) and at low concentration (1 ppm). Thus, metrafenone represents an exceptional combination of protective, eradicative and curative performance, which offers a new approach of effective control against powdery mildew fungi.

The morphological analysis shows that the mode of action of metrafenone differs from traditional and commercialized fungicides. A direct treatment of *B. graminis* with metrafenone induced swelling, bursting and collapse of the hyphal tips. This put forward that the fungicide might weaken the cell wall by affecting cell wall synthesis or, alternatively, by disrupting the apical cap of the actin cytoskeleton (Opalski et al. submiited). Actin and cell wall synthesis act in concert to maintain apical integrity and regulate tip morphology and growth (Wessels, 1990; Jackson and Heath, 1993a; Bartnicki-Garcia, 1999; Torralba and Heath, 2001). In parallel, we tested the effect of polyoxin D, an inhibitor of cell wall synthesis and cytochalasin D, an inhibitor of actin polymerization. The effect of metrafenone was similar to that of cytochalasin D. Swelling of the hyphal tips due to cytochalasin D treatment has been reported in several filamentous fungi (Heath, 1995; Grove and Sweigard, 1996; Torralba et al., 1998b, Bachewich and Heath, 1998). Although polyoxin D similarly caused swelling and bursting of the apex, the chitin synthesis inhibitor did not induced other typical effects of metrafenone. The actin cytoskeleton plays an important role in targeting of vesicles containing cell wall material to hyphal tips (Geitmann and Emons, 2000). Metrafenone might affect the actin in the apex, which is believed to be required for reinforcement of the expanding apical wall in growing hyphae. Moreover, hyphal tips and secondary appressoria

were frequently bifurcated after treatment with metrafenone, suggesting that cell polarity was affected, which might be explained by disturbance of the cytoskeleton or Spitzenkörper (Geitmann and Emons, 2000; Torralba and Heath, 2001; Riquelme and Bartnicki-Garcia, 2004). Benomyl and cytochalasin D caused the splitting of the apex of hyphae. Similarly, cytochalasin A elicited dichotomous branching in *A. nidulans* (Riquelme et al., 2003). Additionally, hyperbranching was observed after treatment with metrafenone, cytochalasin D and benomyl. In the hyphae of *Saprolegnia ferax*, disruption of the actin with latrunculin B also caused hyperbranching (Bachewich and Heath, 1998). However, anti-microtubule drugs typically induced distortion of hyphae as shown for *Aspergillus nidulans* and *Neurospora crassa* (Riquelme et al., 1998; 2003). This was not observed after treatment with metrafenone, and thus we can likely exclude that metrafenone affects the microtubules. This is further supported by the observation that microtubule patterns were only changed in *Bgh* after metrafenone treatment (Opalski et al. submitted). This argues in favor of that metrafenone caused the abnormal branching rather by disturbing the actin cytoskeleton.

Conidiophores and some hyphae showed irregular septation after metrafenone treatment. This suggested that mitosis proceeded without septum formation, indicating that cytokinesis was impaired. Cytochalasin D, benomyl and polyoxin D also induced irregular septation. In filamentous fungi, actin and microtubules are known to play a central role in septum formation and cytokinesis (Harris and Hamer, 1993; Momany and Hamer, 1997). In yeast, the primary septum that separates mother from daughter cells, are mostly composed of chitin (Mc Cullough, 1992). Polyoxin D inhibits septum formation in *Candida albidans* (Becker et al. 1983). In yeast, actin is seen at the neck during formation of the chitin ring and thought to guide vesicles bearing cell wall material to the growing bud (reviewed by Lew and Reed, 1995). Thus, the irregular septation could be due to an effect of the fungicide on actin.

Metrafenone is known to be specific to powdery mildew fungi. After application of metrafenone on non-target *A. alternata* and *S. tritici*, the fungi transiently showed swelling of

cells followed by a normal growth. On the other hand, *A. gossypii* presented subapical bursting in some hyphae, even if metrafenone did not have effect on its development. This observation might indicate that the fungi succeeded to metabolize metrafenone.

Together, metrafenone is a systemic fungicide, specific to powdery mildew fungi, and its mode of action is different from that of traditional fungicides used against *B. graminis*. The comparison of metrafenone with specific inhibitors showed that the fungicide had an effect most similar to cytochalasinD.

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3 Discussion

3.1 Actin polarization, a crucial process in anti-fungal defense

Host actin remodeling was observed during attempts by fungi to penetrate a variety of plants (Gross et al., 1993; Kobayashi et al., 1994; McLusky et al., 1999; Kobayashi and Hakuno, 2003; Takemoto et al., 2003; Xu et al., 1998; Schmelzer, 2002). This suggested that the actin remodeling and cell polarity are crucial in anti-fungal defense. The aim of the present work was to investigate the actin reorganization in epidermal cells of susceptible *Mlo* and resistant *mlo5* barley attacked by *Bgh* as well as to judge the influence of the small G- protein RACB on actin reorganization in this interaction. I could provide clear evidence for differential actin reorganization in barley epidermal cells either being accessible or inaccessible to *Bgh*.

3.1.1 Actin polarization in fungal resistance

The penetration resistance is linked with localized cell wall fortifications and polarized deposition of defense-related compounds beneath sites of fungal attack (Bushnell and Bergquist, 1975; Zeyen et al., 2002). Plant actin cytoskeleton was previously reported to play an important role in the early defense responses of plant cells against fungal penetration (Kobayashi et al., 1992, 1997a, 1997b; Schmelzer, 2002). In this study, the frequency of actin filament (AF) patterns in cells attacked by *Bgh* was compared in susceptible *Mlo* and resistant *mlo5* plants. The results revealed that strong actin polarization to the sites of fungal attack was closely associated with successful penetration resistance, whereas weak actin polarization was connected with successful haustorium formation. At 14-36 hai, the incidence of strong AF focusing to the site of fungal attack was much higher in resistant *mlo5* than in susceptible *Mlo* barley epidermal cells (Figure 3b). Vice versa, treatment of barley cells with the actin polymerization inhibitor, cytochalasin inhibits callose deposition and papilla formation and permit non-pathogenic fungi to penetrate into non-host epidermal cells (Kobayashi et *al*. 1997a, 1997b). We also inhibited the actin polymerization by infiltration of cytochalasin A into *Mlo* and *mlo5* barley. Subsequent, actin staining showed disruption of the AFs after infiltration of cytochalasin A (Figure 1). Treatment with 20 μg. mL^{-1} cytochalasin A resulted in an increase of penetration efficiency by 22% in *Mlo* plants when compared to controls, and partial breakdown of the complete *mlo5* mediated penetration resistance (Supplement, Figure A). These results indicate that the polarized and filamentous state of actin is necessary for barley to block fungal penetration.

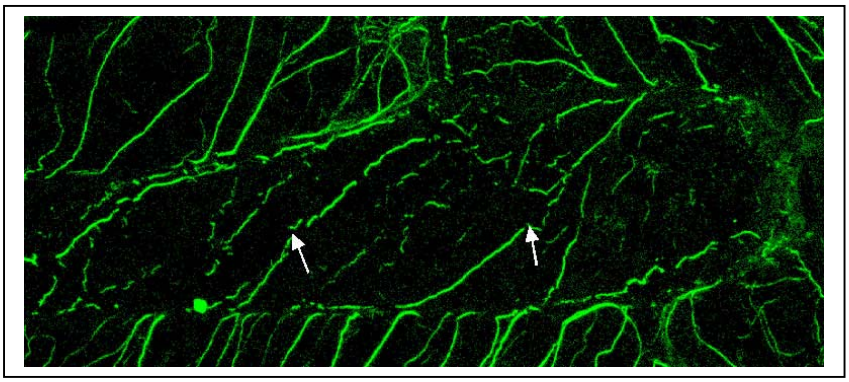

Figure 1. Distribution of actin filaments in a barley epidermal cell after intercellular infiltration of 20 µg. ml^{-1} cytochalasin A. Actin filaments (arrows) are disrupted by cytochalasin A. Actin was stained by Alexa Fluor ® 488 phalloidin (green) and imaged by CLSM microscopy.

The link between strong polarization of AFs and successful penetration resistance is not completely clear. It is tempting to speculate that vesicles contribute to cell wall apposition CWA and that the AFs serve as transport routes for vesicles containing the cell wall compounds. Previously, it was shown that in *mlo5* plants, successful penetration defense is associated with earlier and stronger H_2O_2 accumulation in CWAs and in surrounding large vesicles (Hückelhoven et al., 1999, 2000; Piffanelli et al., 2002). Once reinforced by oxidative cross-linking with H_2O_2, CWAs should be highly resistant to cell-wall-degrading enzymes (Thordal-Christensen et al., 1997; Hückelhoven and Kogel, 2003). Thus, actin could alternatively recruit a cell wall synthetic oxidative machinery to a specific region of the plasma membrane (PM). It was reported that the rearrangement of actin microfilaments was linked to vesicle delivery at the PM at wound sites (Foissner et al., 1996). In tip- growing root hairs, dynamic F-actin meshwork defines the targeting of exocytosis machineries (de Ruijter and Esmons, 1999). Therefore, the cytoskeleton could be involved in directing secretory vesicles to the site of cell wall fortification. Additionally, the *Ror2* gene has been recently isolated from barley and encodes a SNARE (syntaxin). The SNAREs are conserved in animals and plants and control intracellular vesicle targeting (Bock et al., 2001). The ROR2-SNARE, which is required for penetration resistance to *Bgh* seems to be involved in vesicle membrane dynamics (Collins et al., 2003). Consequently, in powdery mildew-resistant *mlo5* barley, the actin cytoskeleton might provide an efficient route for the transport of structural and chemical defense compounds to the site of attempted penetration. Remarkably, the high strong actin polarization incidence took place earlier in *mlo5* than in *Mlo* plants (Figure 3b,

Opalski et al, 2005). In the race between the fungus and the plant, the timing of CWA formation is potentially critical and may suffice to affect the frequency of fungal penetration. Von Röpenack et al. (1998) reported that phenol cross-linking in CWAs occurs earlier in *mlo5* than in *Mlo* plants suggesting that accelerated CWA formation is an important component in *mlo* dependent resistance. Thus, early and strong AF remodeling might contribute to a higher speed of effective papilla formation.

Moreover, at very early stage of infection, epidermal cells show few AF bundles appearing between the nucleus and the penetration site (Figure 2). Later, CWAs were frequently accompanied by the nucleus in non- penetrated cells. Nucleus was also reported to move to penetration site within a transvacuolar cytoplasmic bridge (Gross et al., 1993; Schmelzer, 2002). Hence, AFs possibly direct the nucleus close to the fungal penetration site for shortening signal transduction routes for gene expression changes.

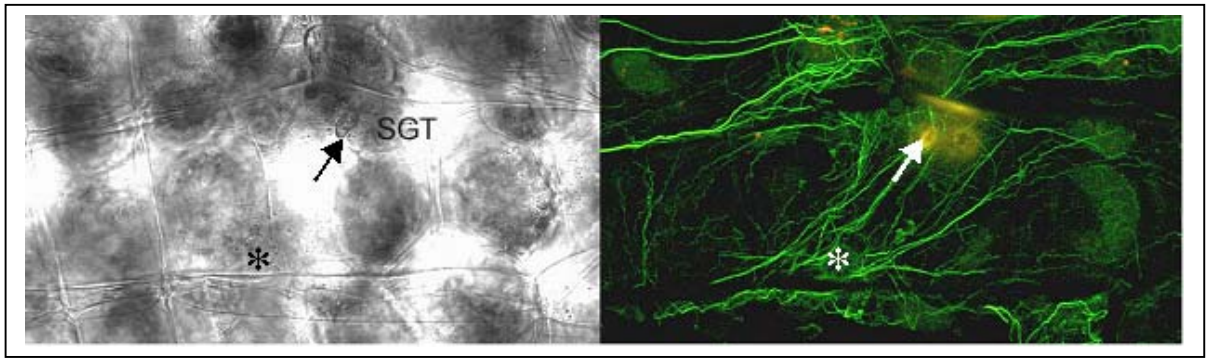

Figure 2. Distribution of actin filaments in resistant *mlo5* barley epidermal cell during attack by *Blumeria graminis* f. sp. *hordei* at 6 hai. Actin was stained by Alexa Fluor ® 488 phalloidin (green) and imaged by CLSM microscopy. The fungal appressorium (SGT: secondary germ tube, arrow) and nucleus (star) are shown by transmission imaging.

The results support the hypothesis that actin plays a central role in determination of the failure of fungal penetration in plant cells. Thus effective penetration resistance is dependent on the actin polarization. However, it is not clear at present whether the plant cytoskeletal responses to fungal attack is stimulated by fungal elicitors, by plant cell wall degradation products, or by the physical pressure of appressorium adhesion or penetration peg protrusion. A mutant of *Magnaporthe,* which is unable to form a penetration peg still elicits actin rearrangement (Xu et al., 1998). The signal, which initiates the actin polarization to the penetration site might

emanate from appressorium on the cell surface or from an earlier stage of fungal development.

A hypothetical model is proposed to illustrate the cell polarization and papilla formation upon fungal infection (Figure 3). At the pre-penetration stage, the epidermal cell senses the developing fungus on its surface and initiates actin polarization to the site of attempted fungal penetration. This potentially involves establishment of a polar site or actin nucleation site by a cortical cue. AF bundles connect the nucleus and the penetration site. During attempted fungal penetration, AFs radiate towards the penetration site. Actin filaments provide a route for the transport and secretion of vesicles to the site of wall fortification. The CWA is formed at the penetration site by massive local cell wall thickening. The nucleus moves along actin bundles to the penetration site and completes the cell polarization.

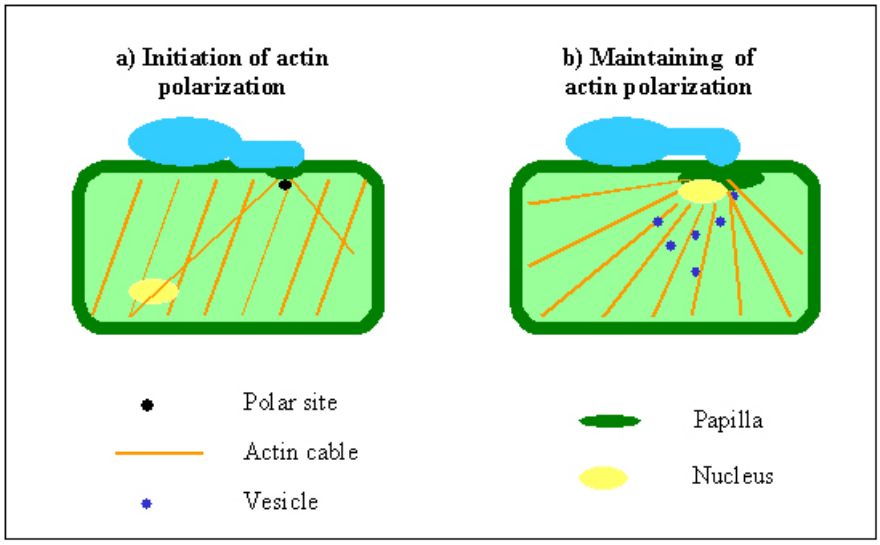

Figure 3. Schematic representation of cell polarization and papilla formation in epidermal cell of barley attacked by *Blumeria graminis* f. sp. *hordei*. (a) At the prepenetration stage, the polar site is selected beneath secondary germ tube, and the epidermal cell initiates actin polarization. (b) Strengthening and maintaining of actin filament polarization occur during attempted fungal penetration. AFs radiate towards the penetration site. The nucleus is translocated. AFs provide a route for the transport of vesicles to the site of wall fortification. Massive local cell wall thickening indicates CWA formation.

3.1.2 Actin organization in penetrated cell

3.1.2.1 MLO modulates actin reorganization

Successful haustorium formation was connected with weak actin polarization in the penetrated cells. Accordingly, the frequency of strong actin focusing towards sites of attempted penetration was lower in susceptible *Mlo*- than in resistant *mlo5* plants. MLO appears to inhibit or to delay actin polarization towards the site of fungal penetration. The delay of the actin polarization could result in less secreted vesicles containing the antifungal compounds at the penetration site and thus might give the fungus enough time to detoxify vesicle contents.

On the other hand, some subtle AFs closely followed the haustorium when it invaginated the plasma membrane. During symbiotic, mycorrhizal interactions in tobacco roots, actin filaments followed the fungal branches and enveloped the arbuscule by a dense network (Genre and Bonfante, 1998). In a symbiosis-defective mutant of *Lotus japonicus*, cytoskeleton disorganization and cell death in the presence of a mycorrhizal fungus was observed suggesting a host factor controlling both at once (Genre and Bonfante, 2002). Parniske (2000) discussed further striking similarities between mycorrhizal and obligate parasitic interactions. Since actin filaments are linked to the plasma membrane via actin-binding proteins (Baluška et al., 2003), actin remodeling could be involved in plasma membrane invagination required for the internalization of a parasitic haustorium. The observation of host actin rings at the tip of haustoria suggests that the host actively takes part in haustorium establishment (Opalski et al., 2005, Figure 2d). Thus, *Bgh* could induce its entry into the plant cell by subversion of host-cell cytoskeleton.

Additionally, the analysis of the time course of strong actin polarization has shown the maintenance of high polarization until 36 hai in *mlo5* plants, whereas in *Mlo* plants, strong polarization declined after fungal penetration (Figure 3b, Opalski et al, 2005). In infected *Mlo* plant cells, the actin cytoskeleton seems to depolarize after the initiation of haustorium development. Kobayashi et al. (1994) reported a similar observation with microtubules of flax cells infected by flax rust. The analysis of microtubule arrays during the infection process indicated that the disappearance of microtubules in the cells does not depend on the time after inoculation but on the development of haustoria within the mesophyll cells. Hence, filamentous actin depolarization might depend on the development of a haustorium within a cell. The depolarization might be also activated by the fungus.

However, MLO is not a general inhibitor actin polarization. Beneath fungal primary germ tubes at an early interaction stage before the formation of appressorium, no difference could

be detected between *MLO* and *mlo5* plants. This can be explained by the fact that *Bgh* induces *Mlo* gene expression and MLO protein accumulation between 6 and 12 hai (Piffanelli et al., 2002). Alternatively, *Bgh* might take over control of the host cytoskeleton by MLO effectors that are released from the appressorium but not from primary germ tubes. Schulze- Lefert and Panstruga (2003) and Hückelhoven and Kogel (2003) suggested the intriguing possibility that *Bgh* might target MLO to suppress host defense. Schulze- Lefert (2004) also reported that *Bgh* might target MLO to suppress/delay the ROR2-SNARE controlling the transport of vesicle containing ROIs, which may provide time for detoxification of vesicle cargo by the fungus. Collectively, the data indicate that functional MLO is required for *Bgh* to modulate polarity in susceptible barley and that such modulation might be part of the fungal defense suppression mechanism.

3.1.2.2 RACB modulates actin reorganization

Small GTP- binding proteins of the RAC family were suggested to act as molecular switches, which regulate actin organization in plant cells (Fu and Yang, 2001). Polarity development is divided into three hierarchical steps: polar site selection by internal or external cues, establishment of the polar site and maintenance of polar sites associated with polar growth. Recent studies suggest that a single type of RAC/ROP GTPases govern all three processes of cell polarity development in plants (Molendijk et al., 2001; Jones et al., 2001). RAC/ROPs were reported to regulate both the formation of a tip- focused Ca^{2+} gradient and actin dynamics, both of which are essential cellular activities for pollen tube and root hair growth (Li et al., 1999; Fu et al., 2001). The selection of the polar site is determined by a cue, probably from the cross-wall in root hair development, whereas the pollen germination is initiated by an external signal from papilla (Fu and Yang, 2001). After the establishment of polarity, the polar tip growth is initiated. During the polar growth, Ca^{2+} gradient and AFs focus to the tip. A model (by Fu and Yang, 2001) is proposed to show the ROP control of cell polarity in root hairs and pollen tubes (Figure 4). The model shows initiation and maintenance of polarity with astonishing similarities to what was observed in barley cells during fungal defense.

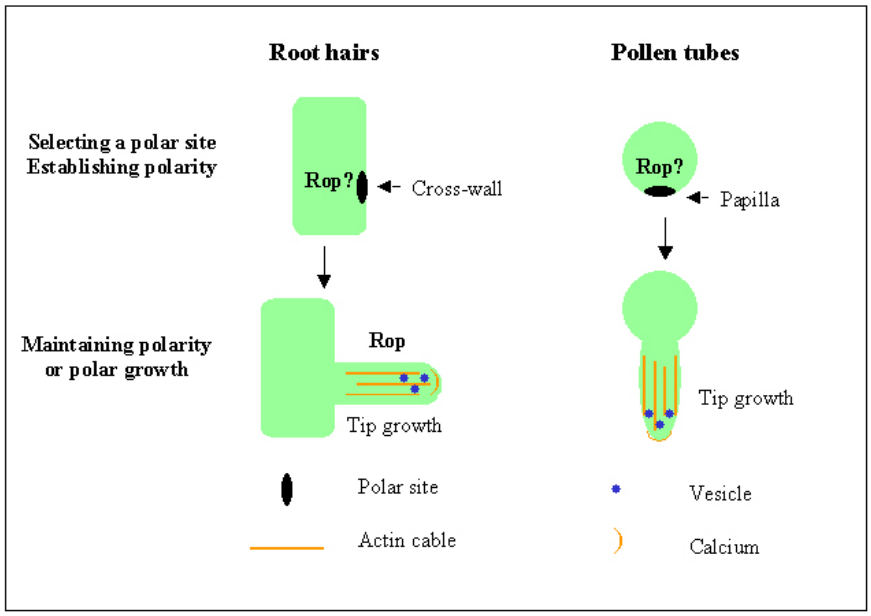

Figure 4. Model of ROP-mediated control of cell polarity development in root hairs and pollen tubes (model changed after Fu and Yang, 2001).

In pollen tubes and root hairs, it was also reported that constitutive activation of RAC/ROPs led to depolarized instead of polarized growth with altered actin cytoskeleton organization (Jones et al., 2002; Molendijk et al., 2001; Gu et al., 2003; Kost et al., 1999; Li et al., 1999; Fu et al., 2001; Tao et al., 2002; Cheung et al., 2003). In CA RACB-expressing cells, we detected a reduced of frequency of AFs focusing towards sites of attempted penetration in both *Mlo* and *mlo5* plants, whereas after RACB knockdown, we observed that the incidence of strong actin polarization increased in *Mlo* barley (Figure 4e, Opalski et al, 2005). A direct role of RACB in actin dynamics is supported by the observation that AF polarization could be partially inhibited in *mlo*-barley by CA RACB overexpression, although resistance remained unaffected (Opalski et al., 2004, and data not shown and Schultheiss et al., 2003). This demonstrated that actin depolarization is not sufficient to break *mlo*-mediated resistance. In contrast to depolarization, the inhibition of actin polymerization into *mlo*- barley appeared sufficient to break complete penetration resistance (Supplement, Figure A). This suggests that RACB function in susceptibility requires functional MLO.

The mechanism of RAC/ROPs dependent regulation of actin cytoskeleton is still not completely understood. Several possibilities are proposed to explain how RAC might control actin dynamic (Figure 5). In pollen tube of *Arabidopsis*, it was reported that Rop GTPase-

regulates tip-focused Ca^{2+} influx and gradient (Li et al., 1999). The tip-localized Ca^{2+} gradient promotes actin dynamics and polarization (Gu et al., 2004). Furthermore, a ROP protein from *Arabidopsis* was suggested to activate the NADPH oxidase. The resultant ROIs then could activate hyperpolarization-activated Ca^{2+} channels to facilitate Ca^{2+} influx for root hair growth, where it is involved in the modulation of actin dynamic (Foreman et al., 2003). There is indirect evidence for Ca^{2+} influx into barley cells attacked by *Bgh* because apoplastic [Ca^{2+}] decreases in susceptible barley attacked by *Bgh* when the fungus attempts to penetrate (Felle et al., 2004). Moreover, functional MLO depends on Ca^{2+}-mediated calmodulin binding to completely perform its role in susceptibility to *Bgh* (Kim et al., 2002). Therefore, we speculate that RACB might be involved in Ca^{2+} ion flux regulation and establishment of subcellular Ca^{2+} gradients required for both focal actin remodeling and Ca^{2+}-dependent antagonism by MLO function (Figure 5).

CA RACB overexpression might induce diffuse instead of focal Ca^{2+} influx, which is amplified by the overproduction of superoxide, which activates the Ca^{2+} channels. Since the Ca^{2+} gradient is involved in the modulation of actin dynamics, this diffuse Ca^{2+} influx might interfere with polarization (Figure 5). Conversely, RACB knockdown might reduce the Ca^{2+} influx, which should limit MLO function in susceptibility.

Furthermore, RACB might regulate a barley actin depolymerization factor (ADF) required for actin remodeling during apoplastic defense. This appears possible because tobacco NtRAC1 regulates NtADF1 indirectly via its phosphorylation status (Chen et al., 2003) suggesting that constitutive NtRAC1 activity leads to over-polymerization of the actin cytoskeleton, which inhibits actin dynamics.

Concurrently, CA RACB might increase the ration of phosphorylated to nonphosphorylated barley ADFs, which leads to over-polymerization of actin cytoskeleton and hence inhibition of actin dynamic (Figure 5). Our results support this hypothesis because CA RACB expressing cells were unable to remodel their actin cytoskeleton to form a focused AF pattern during attack by *Bgh.* Vice versa, RACB knockdown would led to little phosphorylated ADF that should be active in actin remodeling and establishment of polarity.

Collectively, these observations show that RAC signaling coordinates several interacting pathways in its control of actin dynamics during the plant-pathogen interactions (Figure 5).

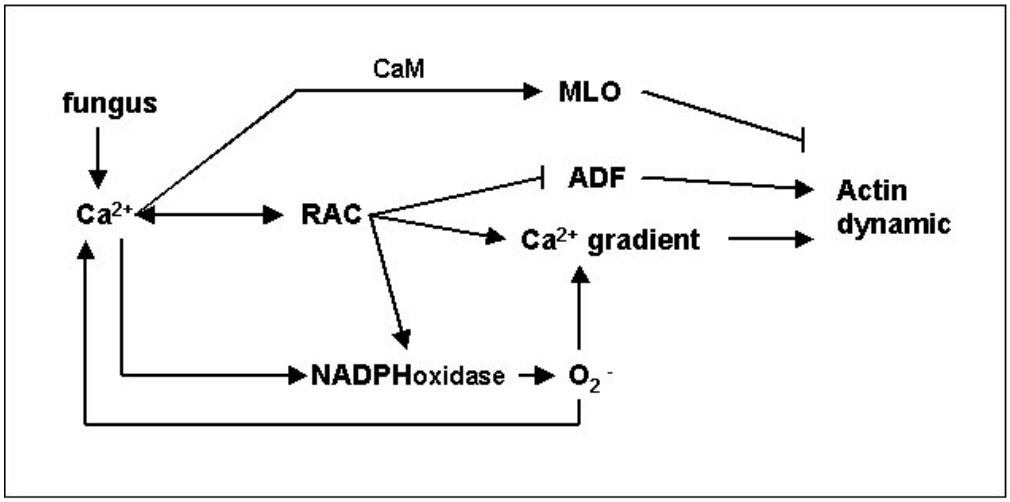

Figure 5. Hypothetical model of RAC-mediated orchestration of multiple interacting downstream and upstream pathways during the barley- *Blumeria graminis* f. sp. *hordei* interaction. Calmodulin (CaM), Actin Depolarization Factor (ADF)

The penetration of host cells by haustoria requires plasma membrane (PM) invagination of host cell (Figure 6). The actin reorganization around haustoria and actin polarization zones close to haustorium tips supports the idea that the host participates for the internalization of pathogens (Figure 6). This process is analogous process to localized cell growth (in the reverse direction to the localized outgrowth that occurs, for example, during root-hairs or pollen tubes formation), which is known to require ROP signaling. We named this phenomenon “inverted tip growth” (Schultheiss et al., 2003).

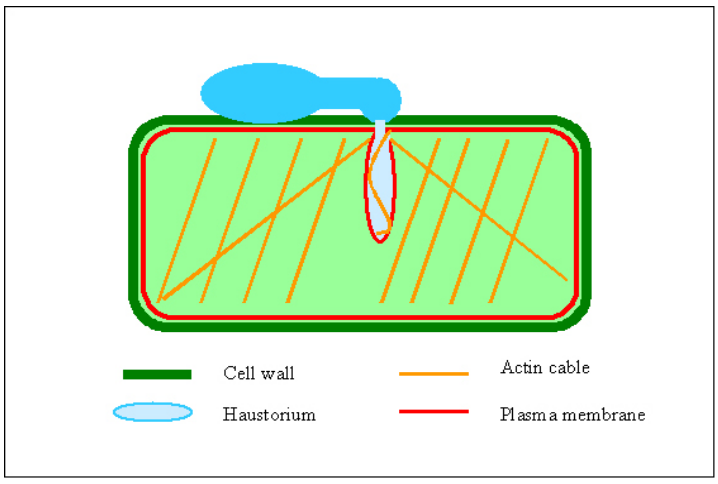

Figure 6. Schematic representation of the actin organization in epidermal cell of barley during haustorium formation of *Blumeria graminis* f. sp. *hordei*. Only few transversal actin filaments radiate towards the penetration site. Microfilaments closely follow the haustorium, which is surrounded by the invaginated host membrane forming an "inverted tip growth".

In animal systems, the invagination processes of the PM such as macropinocytosis and uptake of bacterial pathogens are dependent on small G proteins (e.g. Knodler et al., 2001; Symons and Rusk, 2003). Therefore, RACB might be involved in PM invagination allowing haustorium establishment. In contrast, RACB knockdown possibly cuts such a process down. Concurrently, effector proteins of virulent *Bgh* might overactivate RACB, which leads to inhibition of host actin cytoskeleton dynamics and hence polar defense, finally allowing access to epidermal cells by invagination of PM.

Together, MLO and RACB proteins are involved in signaling for host actin cytoskeleton remodeling and cell polarity during defense against an invading fungal pathogen. They might be corrupted by *Bgh* to induce accessibility of attacked cells.

3.2 Mode of action of Metrafenone

Control of *B. graminis* in cereal is mainly achieved by fungicide treatment, but it is a problem to obtain effective control as the fungal population has the potential to develop tolerance against most used fungicides (De Waard et al., 1993). To face problem arising from the development of resistance of *B. graminis*, against the fungicides, BASF industry has provided a novel systemic fungicide metrafenone. The second part of this current work was to study the mode of action of metrafenone on the powdery mildew of barley (*Bgh*) and wheat (*Bgt*).

3.2.1 Influence of metrafenone on *B. graminis* infection

Metrafenone inhibited the formation and the development of different stages of life cycle of powdery mildew fungi. Treatment of barley and wheat leaves with metrafenone resulted in effective protection against both *Bgh* and *Bgt*. The preventive treatment caused significant reduction in germination and prevented the formation of mycelium and sporulation. Although the effect of metrafenone on germination was weaker than that of conventional fungicides such as kresoxim methyl, quinoxyfen and spiroxamine, treatment with metrafenone resulted in more effective prevention against mycelium formation. When metrafenone was applied directly on *B. graminis*, mycelium was greatly reduced for treatment at 8 hpi and 2 dpi. Metrafenone is an efficient eradicative fungicide at very low concentrations, if it is applied early enough after inoculation. Mycelium cell death occurred as early as 1 to 3 h after

metrafenone application. Thus, metrafenone has a significant effect on the fungal survival. Moreover, metrafenone inhibited or delayed the sporulation. The reduced spore production should reduce secondary infections. Hence, metrafenone is an efficient curative fungicide. It was noteworthy that metrafenone had stronger eradicative and curative activities than kresoxim methyl, quinoxyfen and spiroxamine, when it was applied early after inoculation and at low concentrations. Thus, metrafenone represents an exceptional combination of protective, eradicative and curative performance, which offers a new approach of effective control against powdery mildew fungi.

3.2.2 Effect of preventive treatment with metrafenone

On metrafenone-treated leaves, appressoria typically formed 2 or 3 lobes. The fungicide enhanced the number of appressoria giving rise to papillae whilst reducing the proportion that succeeded in forming a haustorium. Metrafenone blocked fungal development beyond the primary appressoria. If fungi succeeded to produce a haustorium, it was generally malformed and encased by callose. It was noteworthy that treatment of conidiospores used for the inoculation involved also reduced germination and increasing of multilobed appressoria. Thus, one can conclude that metrafenone exerts a direct effect on the fungus. Metrafenone might rather delay fungal development, giving the host more time to prevent penetration. This could explain the increase of the number of papilla and multilobed appressoria. It was remarkable that the actin was delocalized in the appressorial lobe. In *Magnaporthe grisea*, *PLS1*, a gene important for the penetration peg, was identified. The potential functions for PLS1 tetraspanin include focusing mechanical force at the appressorium pore and orchestrating the formation of the actin network at the site of peg emergence (Alspaugh et al., 1998; Dean, 1997). Furthermore, polarization of actin cytoskeleton to the site of penetration-hypha formation was suggested to be involved in localized wall modification, which is essential for penetration by *M. grisea* (Bourett and Howard, 1992; Howard, 1994; Xu et al., 1998). Thus, metrafenone might prevent successful fungal penetration of the host cell by disturbing the actin cytoskeleton. Alternatively, it was demonstrated that cellulases are released from appressorial germ tube and may play a role in penetration (Pryce-Jones et al.,1999). Actin is also involved in the transport and secretion of vesicles containing the enzymes (Heath, 1990). Hence, metrafenone might also affect the secretion of enzymes, which degrade the host cell wall and facilitate penetration.

3.2.3 Direct effect of metrafenone on the morphogenesis of *B. graminis*

The reduction of mycelium formation was correlated with rapid collapse of hyphae. Hyphal collapse was associated with swelling and bursting of hyphal tips. Burst hyphal tips released globule of cytoplasm. The cytological analysis indicated that the swelling of hyphal tips could be due to weakening of cell wall at the apex, disturbance of apical vesicle delivery and disruption of F-actin-cap at the hyphal tip. These observations indicate a compromised polar growth and loss of cell polarity. Interestingly, the effect of cytochalasin D on *Bgh* are similar to that of metrafenone. However, an actin polymerization assays indicated that metrafenone did not directly inhibit actin polymerization (date not shown). It seems that metrafenone rather induced the rearrangement of the actin pattern. Hyphal tip growth is a process many many similarities in diverse walled cells such as pollen tubes and root hairs (Heath and Geitmann, 2000). In filamentous fungi, proteins that play an important role in regulating the organization of the actin also include the Ras and Rho GTPases including Rho, Cdc42 and Rac (Tanaka and takai, 1998; Momany, 2002, Harris and Momany, 2004). In *Ashbya gossypii*, the mutant of *Agrho3* also shows hyphal swelling with delocalized actin at the hyphal tips (Wendland and Philippsen, 2001). In *Colletotricum trifolii*, the mutational activation of *Ras* gene exhibited hyphal tips tending to burst (Truesdell et al.,1999). This support the idea that metrafenone might target the process, which promotes the assembly of tip-localized actin.

Moreover, metrafenone frequently caused bifurcation of hyphal tips and secondary appressoria, likely indicating a disturbance of cell polarity maintenance. The treatment of *Bgh* with cytochalasin D induced also the tip splitting. A mutation in the *Neurospora crassa* actin gene results in branching of hyphal tips and alteration of actin at the tip (Virag and Griffiths, 2004). The process of apical branching was suggested to be due to shift of vesicles from the tip to the side initializing the formation of new hyphal outgrowths (Raudaskoski et al., 1994). Similarly, in metrafenone-treated hyphae, the relocalization.of actin might induce an initiation of new polar sites which leads to a new hyphae explaining the bifurcation (Figure 7).

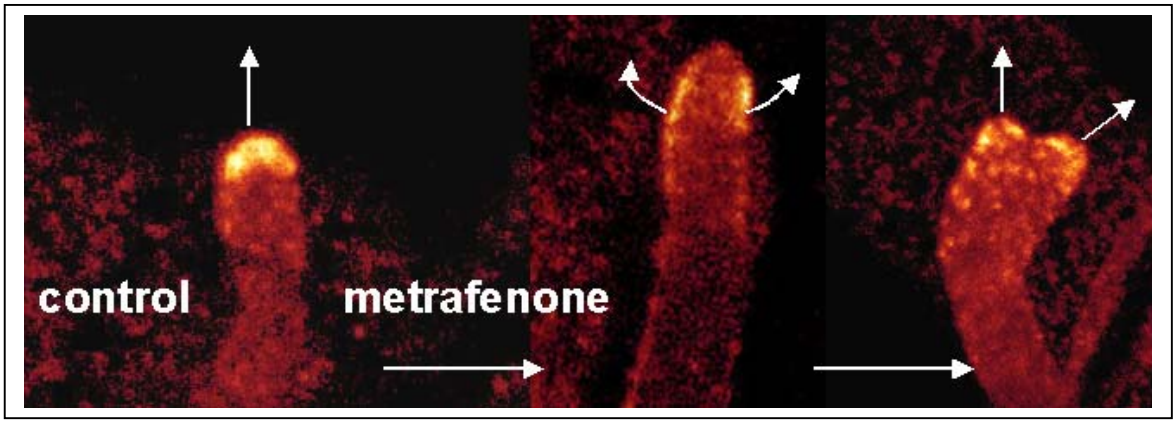

Figure 7. Localization of actin in *Blumeria graminis* f.sp. *hordei* on barley leaves after treatment with 1ppm metrafenone. The actin was stained with anti-actin antibodies and observed by CLSM microscopy.

Moreover, metrafenone treatment resulted in abundant and closely spaced secondary appressoria. Similar anomaly was observed in *Bgh* treated with cytochalasin D. The high number of lateral branches was also observed in hyphae of *Saprolegnia ferax* after application and consecutive removal of latrunculin B (inhibitor of actin polymerization). The phenomenon was preceded by formation of radial arrays of actin in regions without detectable surface protrusion. These sites were consistent with future branches (Bachewich and Heath, 1998). We suggest that in the absence of focused actin at the apex, hyphal growth was not directed and thus cells grew in multiple directions, after treatment with metrafenone.

In the metrafenone-treated hyphae, irregular septation was observed as well. In filamentous fungi, actin has been localized at the sites of septum formation and cytochalasin A was able to block septum formation in *A. nidulans* (Torralba et al., 1998b; Harris et al., 1994). Interestingly, Westfall and Momany (2002) have reported that mutation of septin *AspA* leads to irregular septa and hyperbraching in *A. nidulans*. Localization of AspB at the septation site is dependent on actin. Metrafenone could prevent the formation of septation and induce hyperbranching by disturbance of actin cytoskeleton. The hyperbranching possibly revealed multiple randomly distributed initiation sites of polar growth indicating defects in establishment of cell polarity.

Additionally, in the metrafenone-treated hyphae, multinucleate cells were noticed in the swollen, bifurcated hyphal tips, and region of the numerous secondary appressoria. The presence of multinucleate cells might be due to a nucleus fragmentation, which could be a sign for programmed cell death. Indeed, in *Saccharomyces cerevisiae*, the apoptosis-like cell death starts with DNA fragmentation (Hiramoto et al. 2003).

Concurrently, we checked whether metrafenone could affect microtubules, that are known to be implicated in polarized growth and vesicle transport (Harris and Momany, 2004; Pedregosa et al., 1995). However, IIF microscopy has revealed that metrafenone did not disrupt microtubules. The treatment of *Bgh* with anti-microtubule drug, benomyl, results to certain similar morphological anomalies induced by metrafenone. However, benomyl also induced a typical distortion of the hyphae which was not observed after treatment with metrafenone. Together, this allows to assume that metrafenone does not disrupt the microtubules. Nevertheless, microtubules were often replaced by a "star-bursting-like pattern" in hyphae, after metrafenone application. This reorganization of microtubule was also observed in *S. ferax* after disruption of actin (Bachewich and Heath, 1998). The concomitant alteration in microtubule and actin organization supports postulated F-actin and microtubules interactions (McKerracher and Heath, 1987; Collings et al, 1996). The actin was also suggested to stabilize microtubule organization (Bachewich and Heath, 1998). Therefore, the reorganization of microtubules might be a secondary effect of actin defects.

The inhibition of sporulation was associated with the malformation of conidiophores, that showed multinucleate cells and irregular septation. This suggests that mitosis proceeded without septum formation, indicating that cytokinesis was impaired. Again, actin was delocalized in the tip of the young conidiophores and actin associated with septa was hardly detectable after treatment. Metrafenone did not induced disruption of microtubule in conidiophores. Cytochalasin D induced similar irregular septation of conidiophores. Actin is known to play a central role in septum formation and be involved in cytokinesis (Harris and Hamer, 1993; Prokopenko et al. 2000). It was suggested that Ras and Rho-GTPases play a role in cytokinesis (Prokopenko et al. 2000). Interestingly, the mutation of *Ras* in *C. trifolii* and *NcRas2* in *Neurospora crassa* also results in defects in sporulation (Kana-uchi et al., 1997; Truesdell et al., 2001). Therefore, the fungicide might cause irregular septation and division of nuclei by disturbance of actin organization.

3.2.4 Potential target of metrafenone in *B. graminis*

These results taken together suggest that the mode of action of metrafenone and its potential target are involved in hyphal morphogenesis, polarized hyphal growth, and the establishment and the maintenance of cell polarity in *B. graminis* in wheat and barley. The morphological anomalies induced by the fungicide might be the consequence of the failure to polarize the actin cytoskeleton. The effect of metrafenone on *B. graminis* shows interesting similarities with the deletion of *cflB*, the *Penicillium marneffei* RAC homolog. Δ *cfl B* results cell division

and growth defect in hyphal and conidial cell types such that cells become depolarized and multinucleate, exhibit inappropriate septation, hyperbranching, tip branching and disruption of the actin cytoskeleton (Boyce et al., 2003). Several other similarities of the metrafenone effect with mutation of polarity genes effects suggest that metrafenone might interfere with processes that are essential to establish and maintain cell polarity, which depends on proper polar actin organization.

4. Summary

The biotrophic barley powdery mildew fungus *Blumeria graminis* f. sp. *hordei* (*Bgh*) attacks epidermal cells of barley (Hordeum vulgare L.). *Bgh* invades susceptible barley by penetrating living cells, which remain intact during the period of fungal nourishment and reproduction. Plant cells responding to fungal attack undergo intracellular morphological rearrangements that lead to cell polarization and cell wall apposition formation as the first line of defence. These changes are often mediated by a dynamic cytoskeleton. Staining of the actin cytoskeleton with fluorescence-labelled phalloidin showed that the actin filaments were polarized to the sites of attempted penetration in the resistant host whereas in susceptible hosts, when penetrated, more subtle reorganization took place around fungal haustoria. This suggests a role for host actin remodelling in both penetration resistance and haustorium establishment. Actin focusing was less frequent and seemingly delayed in susceptible wild type barley expressing the susceptibility factor MLO. Additionally, single cell overexpression of a constitutively activated RAC/ROP G-protein, CA RACB, another potential host susceptibility factor and hypothetical actin cytoskeleton regulator, partly inhibited actin reorganization under attack from *Bgh* whereas knockdown of RACB promoted actin focusing. Thus, RACB and potentially, MLO might be involved in the modulation of actin remodelling and cell polarity during defence against fungal pathogen.

Cell polarity appears to play also an important role in the fungal pathogenesis and represents a potential target for new fungicides. The mode of action of a novel systemic benzophenone-derivated fungicide, metrafenone has been analyzed on *Bgh*. It has been revealed that metrafenone represents an exceptional combination of protective, eradicative and curative performance, which offers a new tool of effective control of powdery mildew fungi. Metrafenone was more efficient than most conventionnel fungicides. Metrafenone significantly reduced germination and blocked development beyond formation of appressoria, which showed more lobes and penetrated less often. The fungicide induced swelling, bursting and collapse of the hyphal tips. Hyphal tips and secondary appressoria were also frequently bifurcated. A cytological analysis showed that metrafenone caused disruption of the F-actin cap, weakening of the cell wall at the apex and affection of the apical vesicles. On metrafenone-treated leaves, sporulation was inhibited and the conidiophores presented irregular septation, mutinucleate cells, delocalization of actin. It could be demonstrated that the potential target of metrafenone is involved in hyphal morphogenesis and the initiation and

maintenance of cell polarity. Metrafenone likely disturbs a pathway regulating organization or polarization of the actin cytoskeleton.

5. Zusammenfassung

Der biotrophe Gerstenmehltaupilz *Blumeria graminis* f. sp. *hordei* (*Bgh*) befällt Epidermiszellen von Gerste (*Hordeum vulgare* L.). *Bgh* dringt in anfällige Gerste über Penetration von lebenden Zellen ein. Die Zellen bleiben intakt, während der Pilz sich ernährt und reproduziert. Pflanzenzellen, die auf einen Pilzangriff antworten, durchlaufen intrazelluläre Umgestaltungen, die zur Zellpolarisation und Bildung von Zellwandappositionen als frühe Verteidigunsmaßnahme führen. Diese Änderungen werden oft über ein dynamisches Zytoskelett vermittelt. Färbungen von Aktin mit fluoreszenzmarkiertem Phalloidin zeigten, dass Aktinfilamente in resistenten Wirtspflanzen hin zu Orten der versuchten Penetration polarisiert werden. In zugänglichen Wirten finden bei Penetration subtilere Aktinumgestaltungen um pilzliche Haustorien statt. Dies lässt eine Rolle der Aktinumgestaltung des Wirtes sowohl in Penetrationsresistenz als auch bei Haustorienetablierung vermuten. Die Aktinfokussierung war weniger häufig and scheinbar verzögert in anfälliger Gerste, die den Suszeptibilitätsfaktor MLO exprimiert. Zusätzlich führte Einzelzellüberexpression des konstitutiv aktivierten RAC/ROP G-Proteins, CA RACB, einem weiteren potenziellen Suszeptibilitätsfaktor und hypothetischen Aktinregulator, zu teilweise inhibierter Aktinreorganisation nach Attacke durch *Bgh,* wohingegen „knockdown“ von RACB die Aktinfokussierung förderte. Demnach könnten, während der Abwehr von pilzlichen Pathogenen, RACB und potenziell MLO in die Modulation von Aktinumgestaltung und Zellpolarität involviert sein.

Zellpolarität scheint auch eine wichtige Rolle in der Pathogenese des Pilzes zu spielen und stellt ein mögliches Ziel für neue Fungizide dar. Die Wirkungsweise eines neuen systemischen, von Benzophenon abgeleiteten Fungizides, Metrafenone, wurde in *Bgh* analysiert. Es wurde gezeigt, dass Metrafenone eine außergewöhnliche Kombination aus schützender, eradikativer und kurativerWirkweise liefert. Dies bietet eventuell neue Ansätze zur effektiven Kontrolle von Mehltaupilzen. Metrafenone war dabei effizienter als herkömmliche Fungizide. Metrafenone reduzierte signifikant die Keimung und blockierte die Pilzentwicklung nach der Bildung von Appressorium, die mehr Lappen aufwiesen und weniger häufig penetrierten. Das Fungizid induzierte Schwellen, Platzen und Kollaps von Hyphenspitzen. Die Hyphenspitzen und sekundäre Appressorien waren auch häufig gegabelt. Die zytologische Analyse zeigte, dass Metaferone zum Zusammenbruch der F-Aktin Kappe, zur Schwächung der Zellwand am Apex und zur Beeinflussung von apikalen Vesikeln führte. Auf Metrafenone behandelten Blättern war die Sporulation inhibiert und Konidiophoren wiesen unregelmäßige Septierung, vielkernige Zellen und Delokalisation von Aktin auf. Es wurde gezeigt, dass der potenzielle Wirkort von Metrafenone in die Morphogenese der Hyphen involviert ist und in Initiierung und Aufrechterhaltung von zellulärer

Polarität. Metrafenone stört wahrscheinlich einen Signalweg zur Regulierung und Polarisation des Aktinzytoskelettes.

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7. Supplementary methods

1 Visualization of microtubules

To visualize microtubules, we used indirect immunofluorescence microscopy with tubulin antibodies. Leaf segments of 5x5 mm size were fixed in 4 % formaldehyde in 25 mM piperazine-*N, N′*-bis (2-ethanesulfonic acid) buffer (PIPES, pH 6,8) with 2 mM EGTA, 2 mM $MgCl_2$ and 0.05 % Tween 20 (w/v), at room temperature for 1 h. After being washed in 25 mM PIPES and in 25 mM phosphate buffer (PB, 4.0 g NaCl, 0.1 g KCl, 0.7 g Na_2HPO_4 $2H_2O$, 0,1 g KH_2PO_4 in 500 ml water, pH 6.8), the leaf segments were rinsed in 25 mM PB, pH 6.5. Then, they were transferred to a solution containing 10 mg/mL driselase (InterSpex Products, Inc, San Mateo, CA). After being rinsed in 25 mM PB, pH 6.5, they were further treated with 0.5 % Triton X-100 in 25 mM PB, pH 6.8, for 1h. The specimens were washed with 25 mM PB, pH 6.8 then with TB buffer (50 mM Tris-HCl, 150 mM NaCl, pH 7.6). Following a rinse cycle, leaf segments were incubated with anti-tubulin antibody (NeoMarkers Fremont, CA), dilued 1:100 (w/v) in antibody diluent solution (DAKO, Carpinteria, CA), at room temperature, for 30 min. Vacuum infiltration was repeated three times for 20 s at 25 mm Hg to promote the infiltration. The specimens were washed two times in TB before incubation in goat anti-mouse IgG Alexa Fluor 488® (Molecular Probes, Leiden, The netherlands) dilued 1:100 (w/v) in antibody diluent, at room temperature, for 1 h. Vacuum infiltration was repeated as described above. Finally, the leaves were rinsed with TB, pH 7.6. The specimens were mounted in TB, pH 7.6 on glass slides and observed by CLSM (excitation 488 nm and emission 505-540 nm). Confocal fluorescence images were recorded on a multichannel TCS SP2 confocal system (Leica Microsystems, Bensheim, Germany).

2. Infilration of cytochalasin A

Cytochalasin A (Sigma) was used to disrupt microfilaments and inhibit the actin polymerization. Cytochalasin A was dissolved in dimethylsulfoxide (DMSO) at 1mg mL^{-1} for the stock solutions. These stock solutions were stored at –20 °C until use and diluted to 20 µg ml^{-1} with distilled water. Cytochalasin A was injected in intercellular space of primary leaves of barley as described Hagborg (1970). In control experiments, we used distilled water.

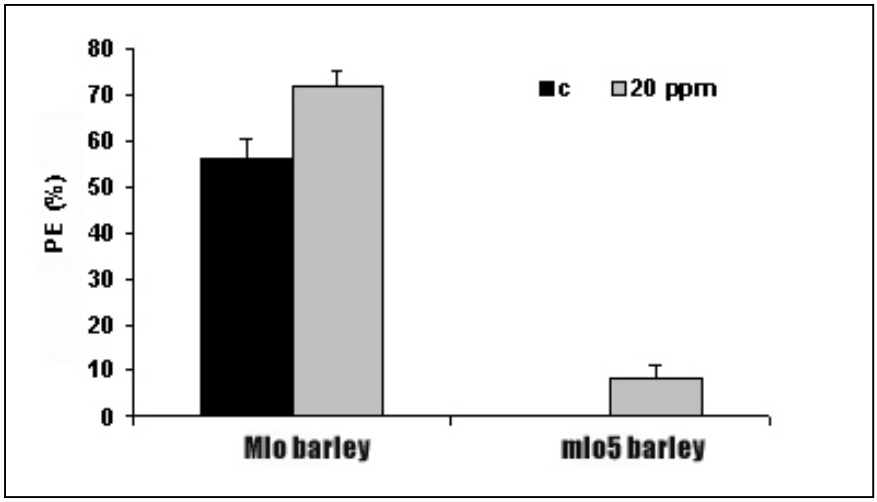

Figure A. Effect of cytochalasin A infiltration in intercellular space of primary leaves of *Mlo* and *mlo5* barley at 8 hai on penetration efficiency of *Blumeria graminis* f. sp. *hordei* at 30 hai. Observations were made on 5 pieces of primary leaf segments counting 100 conidia each. The experiments was repeated 3 times. PE: penetration efficiency; c: control

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